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COTTON PLANT CELLS GENETICALLY ENGINEERED TO EXPRESS INSECTICIDAL BACILLUS THURINGIENSIS CRYSTAL PROTEIN

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(56) Prior Art Documents
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(57) Claim

1. A cotton cell comprising a chimeric gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein, exhibiting toxicity toward Dipteran and Lepidopteran insects.

21. The cell of claim 20 wherein the gene expresses a polypeptide having the amino acid sequence:

Sequence of the formula (II)

Met Asp Asn Asn Pro Asn Ile Asn Glu Cys	10
Ile Pro Tyr Asn Cys Leu Ser Asn Pro Glu	20
Val Glu Val Leu Gly Gly Glu Arg Ile Glu	30
Thr Gly Tyr Thr Pro Ile Asp Ile Ser Leu	40
Ser Leu Thr Gln Phe Leu Leu Ser Glu Phe	50
Val Pro Gly Ala Gly Phe Val Leu Gly Leu	60
Val Asp Ile Ile Trp Gly Ile Phe Gly Pro	70
Ser Gln Trp Asp Ala Phe Leu Val Gln Ile	80
Glu Gln Leu Ile Asn Gln Arg Ile Glu Glu	90
Phe Ala Arg Asn Gln Ala Ile Ser Arg Leu	100
Glu Gly Leu Ser Asn Leu Tyr Gln Ile Tyr	110
Ala Glu Ser Phe Arg Glu Trp Glu Ala Asp	120
Pro Thr Asn Pro Ala Leu Arg Glu Glu Met	130
Arg Ile Gln Phe Asn Asp Met Asn Ser Ala	140
Leu Thr Thr Ala Ile Pro Leu Phe Ala Val	150
Gln Asn Tyr Gln Val Pro Leu Leu Ser Val	160
Tyr Val Gln Ala Ala Asn Leu His Leu Ser	170
Val Leu Arg Asp Val Ser Val Phe Gly Gln	180
Arg Trp Gly Phe Asp Ala Al Thr Ile Asn	190

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Ser Arg Tyr Asn Asp Leu Thr Arg Leu Ile	200
Gly Asn Tyr Thr Asp His Ala Val Arg Trp	210
Tyr Asn Thr Gly Leu Glu Arg Val Trp Gly	220
Pro Asp Ser Arg Asp Trp Ile Arg Tyr Asn	230
Gln Phe Arg Arg Glu Leu Thr Leu Thr Val	240
Leu Asp Ile Val Ser Leu Phe Pro Asn Tyr	250
Asp Ser Arg Thr Tyr Pro Ile Arg Thr Val	260
Ser Gln Leu Thr Arg Glu Ile Tyr Thr Asn	270
Pro Val Leu Glu Asn Phe Asp Gly Ser Phe	280
Arg Gly Ser Ala Gln Gly Ile Glu Gly Ser	290
Ile Arg Ser Pro His Leu Met Asp Ile Leu	300
Asn Ser Ile Thr Ile Tyr Thr Asp Ala His	310
Arg Gly Glu Tyr Tyr Trp Ser Gly His Gln	320
Ile Met Ala Ser Pro Val Gly Phe Ser Gly	330
Pro Glu Phe Thr Phe Pro Leu Tyr Gly Thr	340
Met Gly Asn Ala Ala Pro Gln Gln Arg Ile	350
Val Ala Gln Leu Gly Gln Gly Val Tyr Arg	360
Thr Leu Ser Ser Thr Leu Tyr Arg Arg Pro	370
Phe Asn Ile Gly Ile Asn Asn Gln Gln Leu	380
Ser Val Leu Asp Gly Thr Glu Phe Ala Tyr	390
Gly Thr Ser Ser Asn Leu Pro Ser Ala Val	400
Tyr Arg Lys Ser Gly Thr Val Asp Ser Leu	410
Asp Glu Ile Pro Pro Gln Asn Asn Asn Val	420
Pro Pro Arg Gln Gly Phe Ser His Arg Leu	430
Ser His Val Ser Met Phe Arg Ser Gly Phe	440
Ser Asn Ser Ser Val Ser Ile Ile Arg Ala	450
Pro Met Phe Ser Trp Ile His Arg Ser Ala	460
Glu Phe Asn Asn Ile Ile Pro Ser Ser Gln	470
Ile Thr Gln Ile Pro Leu Thr Lys Ser Thr	480
Asn Leu Gly Ser Gly Thr Ser Val Val Lys	490
Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu	500
Arg Arg Thr Ser Pro Gly Gln Ile Ser Thr	510
Leu Arg Val Asn Ile Thr Ala Pro Leu Ser	520
Gln Arg Tyr Arg Val Arg Ile Arg Tyr Ala	530
Ser Thr Thr Asn Leu Gln Phe His Thr Ser	540
Ile Asp Gly Arg Pro Ile Asn Gln Gly Asn	550
Phe Ser Ala Thr Met Ser Ser Gly Ser Asn	560
Leu Gln Ser Gly Ser Phe Arg Thr Val Gly	570
Phe Thr Thr Pro Phe Asn Phe Ser Asn Gly	580
Ser Ser Val Phe Thr Leu Ser Ala His Val	590
Phe Asn Ser Gly Asn Glu Val Tyr Ile Asp	600
Arg Ile Glu Phe Val Pro Ala Glu Val Thr	610
Phe Glu Ala Glu Tyr Asp Leu Glu Arg Ala	620
Gln Lys Ala Val Asn Glu Leu Phe Thr Ser	630
Ser Asn Gln Ile Gly Leu Lys Thr Asp Val	640
Thr Asp Tyr His Ile Asp Gln Val Ser Asn	650
Leu Val Glu Cys Leu Ser Asp Glu Phe Cys	660
Leu Asp Glu Lys Lys Glu Leu Ser Glu Lys	670
Val Lys His Ala Lys Arg Leu Ser Asp Glu	680
Arg Asn Leu Leu Gln Asp Pro Asn Phe Arg	690
Gly Ile Asn Arg Gln Leu Asp Arg Gly Trp	700
Arg Gly Ser Thr Asp Ile Thr Ile Gln Gly	710
Gly Asp Asp Val Phe Lys Glu Asn Tyr Val	720
Thr Leu Leu Gly Thr Phe Asp Glu Cys Tyr	730
Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu	740
Ser Lys Leu Lys Ala Tyr Thr Arg Tyr Gln	750

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Leu	Arg	Gly	Tyr	Ile	Glu	Asp	Ser	Gln Asp 760
Leu	Glu	Ile	Tyr	Leu	Ile	Arg	Tyr	Asn Ala 770
Lys	His	Glu	Thr	Val	Asn	Val	Pro	Gly Thr 780
Gly	Ser	Leu	Trp	Pro	Leu	Ser	Ala	Pro Ser 790
Pro	Ile	Gly	Lys	Cys	Ala	His	His	Ser His 800
His	Phe	Ser	Leu	Asp	Ile	Asp	Val	Gly Cys 810
Thr	Asp	Leu	Asn	Glu	Asp	Leu	Gly	Val Trp 820
Val	Ile	Phe	Lys	Ile	Lys	Thr	Gln	Asp Gly 830
His	Ala	Arg	Leu	Gly	Asn	Leu	Glu	Phe Leu 840
Glu	Glu	Lys	Pro	Leu	Val	Gly	Glu	Ala Leu 850
Ala	Arg	Val	Lys	Arg	Ala	Glu	Lys	Lys Trp 860
Arg	Asp	Lys	Arg	Glu	Lys	Leu	Glu	Trp Glu 870
Thr	Asn	Ile	Val	Tyr	Lys	Glu	Ala	Lys Glu 880
Ser	Val	Asp	Ala	Leu	Phe	Val	Asn	Ser Gln 890
Tyr	Asp	Arg	Leu	Gln	Ala	Asp	Thr	Asn Ile 900
Ala	Met	Ile	His	Ala	Ala	Asp	Lys	Arg Val 910
His	Ser	Ile	Arg	Glu	Ala	Tyr	Leu	Pro Glu 920
Leu	Ser	Val	Ile	Pro	Gly	Val	Asn	Ala Ala 930
Ile	Phe	Glu	Glu	Leu	Glu	Gly	Arg	Ile Phe 940
Thr	Ala	Phe	Ser	Leu	Tyr	Asp	Ala	Arg Asn 950
Val	Ile	Lys	Asn	Gly	Asp	Phe	Asn	Asn Gly 960
Leu	Ser	Cys	Trp	Asn	Val	Lys	Gly	His Val 970
Asp	Val	Glu	Glu	Gln	Asn	Asn	His	Arg Ser 980
Val	Leu	Val	Val	Pro	Glu	Trp	Glu	Ala Glu 990
Val	Ser	Gln	Glu	Val	Arg	Val	Cys	Pro Gly 1000
Arg	Gly	Tyr	Ile	Leu	Arg	Val	Thr	Ala Tyr 1010
Lys	Glu	Gly	Tyr	Gly	Glu	Gly	Cys	Val Thr 1020
Ile	His	Glu	Ile	Glu	Asn	Asn	Thr	Asp Glu 1030
Leu	Lys	Phe	Ser	Asn	Cys	Val	Glu	Glu Glu 1040
Val	Tyr	Pro	Asn	Asn	Thr	Val	Thr	Cys Asn 1050
Asp	Tyr	Thr	Ala	Thr	Gln	Glu	Glu	Tyr Glu 1060
Gly	Thr	Tyr	Thr	Ser	Arg	Asn	Arg	Gly Tyr 1070
Asp	Gly	Ala	Tyr	Glu	Ser	Asn	Ser	Ser Val 1080
Pro	Ala	Asp	Tyr	Ala	Ser	Ala	Tyr	Glu Glu 1090
Lys	Ala	Tyr	Thr	Asp	Gly	Arg	Arg	Asp Asn 1100
Pro	Cys	Glu	Ser	Asn	Arg	Gly	Tyr	Gly Asp 1110
Tyr	Thr	Pro	Leu	Pro	Ala	Gly	Tyr	Val Thr 1120
Lys	Glu	Leu	Glu	Tyr	Phe	Pro	Glu	Thr Asp 1130
Lys	Val	Trp	Ile	Glu	Ile	Gly	Glu	Thr Glu 1140
Gly	Thr	Phe	Ile	Val	Asp	Ser	Val	Glu Leu 1150
Leu	Leu	Met	Glu	Glu	End			1156

38. A method of producing transformed, embryogenic cotton callus which comprises:

a) contacting a cotton explant with an *Agrobacterium* vector containing a chimeric gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein and a gene that confers resistance to an antibiotic on cotton cells, the period of the contacting being sufficient to transfer the genes to the explant;

b) incubating the transformed explant in a callus growth medium for a period of from about 15 to about 200 hours at a temperature of from 25° to about 35°C under a cycle of about 16 hours light and 8 hours dark to develop callus from the explants;

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c) contacting the incubated explants with a callus growth medium containing an antibiotic toxic to *Agrobacterium* for a time sufficient to kill the *Agrobacterium*;

d) culturing the callus free of *Agrobacterium* on a callus growth medium;

e) contacting the resulting embryogenic callus with the antibiotic in a concentration sufficient to permit selection of callus resistant to the antibiotic; and

f) selecting transformed embryogenic callus.

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FORM 10

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PATENTS ACT 1952

COMPLETE SPECIFICATION

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(ORIGINAL)

FOR OFFICE USE:

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Complete Specification Lodged:
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Related Art:

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Complete Specification for the invention entitled:

Insecticidal Cotton Plant Cells

The following statement is a full description of this invention, including the best method of performing it known to me/us

5845/11

5-16768/CGC 1313/CIP

Insecticidal cotton plant cells

Abstract

The present invention is directed to a chimeric gene that expresses in cotton cells insecticides having substantially the insect toxicity properties of the crystal protein produced by *Bacillus thuringiensis*.

The transformed cells are regenerated into plants that are toxic to the larvae of *Leptidoptera*, ~~*Coleoptera*~~ and *Diptera*.

5-16768/CGC 1313/CIP

Insecticidal cotton plant cells

The present invention is directed to a chimeric gene that expresses in cotton cells insecticides having substantially the insect toxicity properties of the crystal protein produced by *Bacillus thuringiensis*.

B. thuringiensis (hereinafter *Bt*) is a species of bacteria that produces a crystal protein, also referred to as δ-endotoxin. This crystal protein is, technically, a protoxin that is converted into a toxin upon being ingested by larvae of lepidopteran, coleopteran and dipteran insects.

The crystal protein from *Bt* is a potentially important insecticide having no known harmful effects on humans, other mammals, birds, fish or on insects other than the larvae of lepidopteran, coleopteran and dipteran insects. The activity of the *Bt* toxin is extremely high, so that only nanogram amounts are required to kill susceptible insect larvae. Other advantages of the use of the crystal protein from *Bt* as an insecticide include its broad spectrum of activity against lepidopteran, coleopteran and dipteran insect larvae, and the apparent difficulty of such larvae to develop resistance against the crystal protein, even where the crystal protein is used on a large scale. Said larvae are a major problem in agriculture and forestry, and especially in cotton cultivation.

The crystal protein is effective as an insecticide when it is applied to plants subject to lepidopteran, coleopteran or dipteran larvae infestation. Such plants include broccoli, lettuce and cotton. Ledidopteran larvae infestation is especially serious in cotton plants.

So far, the *Bt* crystal protein (protoxin) was isolated from the Bacillus and applied to the plants by standard methods such as by dusting or spraying. Preparations containing the *Bt* crystal protein are used commercially as biological insecticides.

For example:
Bactospeine, distributed by Biochem Products Ltd.,
Dipel, distributed by Abbot Laboratories; and
Thurcide, distributed by Sandoz AG.

The fact that *Bt* produces the crystal protein only during sporulation represents a significant disadvantage in connection with the manufacture and use of this biological insecticide. Such growth phase limitation, particularly in an industrial process, can result in inconvenience and excessive time requirements during manufacture. In addition, costs associated with said manufacture made it difficult for such a biological insecticide to compete effectively with other commercially available products based on chemicals, such as, for example, pyrethroid derivatives.

A further disadvantage with respect to the use of the *Bt* toxin is, for example, the fact that the protein usually remains on the surface of the plants being treated, where it is effective only against surface-feeding larvae, and where it is inactivated by prolonged exposure to ultraviolet radiation. This inactivation may be at least one cause of the general lack of persistance of the crystal protein in the environment. Accordingly, frequent and expensive application of the crystal protein is necessary.

These and other disadvantages can be overcome by incorporating and expressing a gene coding for a *Bt* crystal protein or for a protein having substantially the insect toxicity properties of a *Bt* crystal protein in plants. The present invention describes how these disadvantages can be overcome by incorporating and expressing a gene coding for a *Bt* crystal protein or for a protein having substantially the insect toxicity

properties of a *Bt* crystal protein in corn protoplasts and regenerating fertile transgenic corn plants from the transformed protoplasts and culturing these insect resistant corn plants.

By taking advantage of genetic engineering, a gene responsible for the production of a useful polypeptide can be transferred from a donor cell, in which the gene naturally occurs, to a host cell, in which the gene does not naturally occur (U.S. patents 4,237,224 and 4,468,464). There are, in fact, few inherent limits to such transfers. Genes can be transferred between viruses, bacteria, plants and animals. In some cases, the transferred gene is functional, or can be made to be functional, in the host cell. When the host cell is a plant cell, whole plants can be regenerated from the cell.

Genes typically contain regions of DNA sequences including a promoter and a transcribed region. The transcribed region normally contains a 5' untranslated region, a coding sequence, and a 3' untranslated region.

The promoter contains the DNA sequence necessary for the initiation of transcription, during which the transcribed region is converted into mRNA. In eukaryotic cells, the promoter is believed to include a region recognized by RNA polymerase and a region which positions the RNA polymerase on the DNA for the initiation of transcription. This latter region, which is referred to as the TATA box, usually occurs about 30 nucleotides upstream from the site of transcription initiation.

Following the promoter region is a sequence that is transcribed into mRNA but is not translated into polypeptide. This sequence constitutes the so-called 5' untranslated region and is believed to contain sequences that are responsible for the initiation of translation, such as a ribosome binding site.

The coding region is the sequence that is just downstream from the 5' untranslated region in the DNA or the corresponding RNA. It is the coding region that is translated into polypeptides in accordance with the

genetic code. *Bt* for example, has a gene with a coding sequence that translates into the amino acid sequence of the insecticidal protoxin crystal protein.

The coding region is followed downstream by a sequence that is transcribed into mRNA, but is not translated into polypeptide. This sequence is called the 3' untranslated region and is believed to contain a signal that leads to the termination of transcription and, in eukaryotic mRNA, a signal that causes polyadenylation of the transcribed mRNA strand. Polyadenylation of the mRNA is believed to have processing and transportation functions.

Natural genes can be transferred in their entirety from a donor cell to a host cell. It is often preferable however, to construct a gene containing the desired coding region with a promoter and, optionally, 5'and 3' untranslated regions that do not, in nature, exist in the same gene as the coding region. Such constructs are known as chimeric genes.

Genetic engineering methods have been described for improved ways of producing the crystal protein. For example, U.S. patents 4,448,885 and 4,467,036, describe plasmids for producing crystal protein in bacterial strains other than *Bt*. These methods permit production of the crystal protein, but do not overcome the disadvantages of using the crystal protein as a commercial insecticide.

Suggestions have been made to clone *Bt* toxin genes directly into plants in order to permit the plants to protect themselves (Klausner, 1984). The European Patent Application EP-0,142,924 (Agrigenetics) alleges a method for cloning toxin genes from *Bt* in tobacco (page 59) and suggests protecting cotton the same way (page 77).

Such a suggestion constitutes mere speculation, however, until methods for transforming cotton cells and regenerating plants from the cells are available. Such methods are described in U.S. patent application serial no. 122,200 entitled "Regeneration and Transformation of Cotton", assigned to Phytogen, and in U.S. patent application Serial No. 122,162 entitled "An Efficient Method for Regenerating Cotton from Cultured

Cells", assigned to Ciba-Geigy. U.S. patent applications serial no. 122,200 and 122,162 were filed the same day as the present application. The methods for transforming cotton cells in the Phytogen patent application serial no. 122,200 and the methods for regenerating cotton plants in the Phytogen and Ciba-Geigy patent applications serial no. 122,200 and 122,162, respectively, are incorporated herein by reference.

A need exists for developing new methods for producing the crystal protein of *Bt* or a similar polypeptide having substantially the insect toxicity properties (insecticidal activity) of a *Bt* crystal protein in cells of cotton plants and for new methods of controlling insect larvae which feed on said cotton plants. "Controlling" should be understood as referring either to killing the larvae, or at least reducing their feeding. Further need exists for a method for protecting cotton plants against damage caused by pests or pathogens comprising the production of an amount of a pesticidal or antipathogenic protein in the plant cell and plant respectively, which amount is sufficiently effective for killing or controlling the pest or the pathogen. Further need exists preferably for a method for protecting cotton plants against insect damage comprising the production of an insecticidal effective amount of a *Bt* crystal protein or a protein having substantially the insect toxicity properties of a *Bt* crystal protein in the plant cell, which amount is sufficiently effective for killing or controlling the insects which feed on said plants. Further need exists for a method for protecting cotton plants from damage by chemical agents, such as, for example by increasing the tolerability against certain herbicides, so that such chemicals can be safely applied to cotton plants which have minimal side effects on the ecosystem.

These and other objects of the present invention have been achieved by providing chimeric genes capable of expressing in cotton cells a polypeptide having substantially the insect toxicity properties of *Bt* crystal protein (hereinafter, chimeric *Bt* toxin gene), as can be seen from the following detailed description.

The present invention primarily relates to transgenic cotton plant cells having stably incorporated into the plant genome a chimeric gene that expresses in cotton cells a polypeptide having substantially the insect toxicity properties of the *Bt* crystal protein.

Also comprised by the present invention are transgenic cotton plants that can be regenerated from transgenic cotton plant cells and have stably incorporated into their genome said chimeric gene that upon expression renders the cotton plant unattractive and/or toxic to insects so that they stop feeding on the plant.

This invention also relates to the propagules and progeny of said transgenic cotton plants.

Propagules of said transgenic cotton plants include any material that can be sexually or asexually propagated or propagated in-vivo or in-vitro. Among this propagating material protoplasts, cells, calli, tissues, organs, seeds, embryos, pollen, ovules, zygotes or any other propagating material that can be obtained from said transgenic cotton plants are preferred.

A further object of the present invention includes the progeny of said transgenic cotton plant cells or plants.

The progeny of transgenic cotton plants includes mutants and variants thereof. Mutants and variants include, for example, those plants obtained from cell fusion or mutant selection that still have the characteristic properties of the starting material, caused by the previous transformation of exogenous DNA.

It is, therefore, an object of the present invention to provide a method for producing in cotton cells a toxin that has substantially the insect toxicity properties of *Bt* crystal protein. Especially preferred is a method of transforming cotton cells undergoing suspension culture on a callus growth medium which comprises, after a suspension subculture growth cycle,

a) recovering cells and any embryogenic callus from the callus growth medium;

b) resuspending the cells and the embryogenic callus in a callus growth medium containing *Agrobacterium* vector having a gene that confers resistance to the antibiotic hygromycin on cotton cells while maintaining suspension growth conditions for a period of time sufficient to transform the suspended cells;

c) recovering the suspended cells from the callus growth medium containing the *Agrobacterium*;

d) treating the transformed cells and the embryogenic callus with an antibiotic in sufficient concentration to kill the *Agrobacterium*;

e) contacting the cells and the embryogenic callus with the antibiotic hygromycin in order to select the transformed cells and embryogenic callus;

f) filtering the suspension to remove embryogenic callus greater than about 600 microns (μm).

It is a further object of the present invention to provide a method for killing insect larvae by feeding them cotton plant cells containing chimeric genes that express an insecticidal amount of a toxin having substantially the insect toxicity properties of *Bt* crystal protein. The insecticidal cotton plant cells include those from whole plants and parts of plants as well as individual cotton cells in culture.

It is a further object of the present invention to provide a method for protecting cotton plants against insect damage comprising the expression of a *Bt* crystal protein or a protein having substantially the insect toxicity properties of a *Bt* crystal protein in the plant cells constituting the plant, in an amount sufficient to kill or at least to control the insect larvae.

Especially preferred is a method for protecting cotton plants against damage caused by lepidopteran larvae.

It is a further object of the present invention to provide a method wherein the crystal protein or a protein having substantially the insect toxicity properties of a *Bt* crystal protein is expressed in the plant cell in an amount sufficient to render the plant unattractive and/or toxic to insects, so that they stop feeding on the plants.

It is an additional object of the present invention to provide the genes and other DNA segments as well as the cells and plants associated with the above methods.

According to a first embodiment of this invention, there is provided a cotton cell comprising a chimeric gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein, exhibiting toxicity toward Dipteran and Lepidopteran insects.

According to a second embodiment of this invention, there is provided a culture of cotton cells according to the first embodiment.

According to a third embodiment of this invention, there is provided a cotton plant comprising a gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein, exhibiting toxicity toward Dipteran and Lepidopteran insects.

According to a fourth embodiment of this invention, there is provided propagules of a transgenic cotton plant according to the third embodiment.

According to a fifth embodiment of this invention, there is provided progeny of a transgenic cotton plant according to the third embodiment, or mutants and variants thereof, that still have the characteristic properties of the starting material, caused by the previous transformation of exogenous DNA.

According to a sixth embodiment of this invention, there is provided a method of producing transformed, embryogenic cotton tallus which comprises:

a) contacting a cotton explant with an *Agrobacterium* vector containing a chimeric gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein and a gene that confers resistance to an antibiotic on cotton cells, the period of the contacting being sufficient to

transfer the genes to the explant;

b) incubating the transformed explant in a callus growth medium for a period of from about 15 to about 200 hours at a temperature of from 25° to about 35°C under a cycle of about 16 hours light and 8 hours dark to develop callus from the explants;

c) contacting the incubated explants with a callus growth medium containing an antibiotic toxic to *Agrobacterium* for a time sufficient to kill the *Agrobacterium*;

d) culturing the callus free of *Agrobacterium* on a callus growth medium;

e) contacting the resulting embryogenic callus with the antibiotic in a concentration sufficient to permit selection of callus resistant to the antibiotic; and

f) selecting transformed embryogenic callus.

According to a seventh embodiment of this invention, there is provided a method of transforming cotton cells undergoing suspension culture on a callus growth medium which comprises, after a suspension subculture growth cycle:

a) recovering cells and any embryogenic callus from the callus growth medium;

b) resuspending the cells and embryogenic callus in a callus growth medium containing an *Agrobacterium* vector containing a chimeric gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein and a gene that confers resistance to an antibiotic on cotton cells while maintaining suspension growth conditions for a period of time sufficient to transform the suspended cells;

c) recovering the suspended cells from the callus growth medium containing the *Agrobacterium*;

d) treating the transformed cells and the embryogenic callus with an antibiotic toxic to *Agrobacterium* in sufficient concentration and for a time sufficient to kill the *Agrobacterium*;

e) contacting the cells and embryogenic callus with the antibiotic in order to select the transformed cells and embryogenic callus;

f) filtering the suspension to remove embryogenic callus greater than about 600 μm.

According to an eighth embodiment of this invention, there is

provided cotton plants transformed to express a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein and have resistance to the antibiotic hygromycin.

According to a ninth embodiment of this invention, there is provided a method for protecting cotton plants against Dipteran and Lepidopteran insect damage comprising the expression of a *Bt* crystal protein or a protein having substantially the insect toxicity properties of a *Bt* crystal protein in the plant cells constituting the plant, in an amount sufficient to kill or to control the insect larvae.

According to a tenth embodiment of this invention, there is provided a method for killing or controlling Dipteran and Lepidopteran insect larvae by feeding them cotton plant cells containing chimeric genes that express an insecticidal amount of a toxin having substantially the insect toxicity properties of *Bt* crystal protein.

Additional embodiments of the present invention include the chimeric *Bt* toxin gene in vectors, bacteria, plant cells in culture, and plant cells in living plants, as well as methods for producing a toxin having substantially the insect toxicity properties of *Bt* crystal protein in cotton cells and methods for protecting cotton plants against insect damage, comprising the production of a controlling or insecticidal effective amount of a *Bt* crystal protein or a protein having substantially the insect toxicity properties of a *Bt* crystal protein in the plant cell.

Figures:

Figure 1: Construction of mp 19/bt, a plasmid containing the 5' end of the *Bt* protoxin gene.

Figure 2: Construction of mp 19/bt, ca/del, a plasmid containing the CaMV gene VI promoter fused to the 5' end of *Bt* protoxin coding sequence.

Figure 3: Construction of p702/bt, a plasmid having the 3' coding region of the protoxin fused to the CaMV transcription termination signals.

Figure 4: Construction of pBR322/bt 14, containing the complete protoxin coding sequence flanked by CaMV promoter and terminator sequences.

Figure 5: Construction of pRK252/Tn903/BglII.

GSR/LMM/TCW/1160v

Figure 6: Construction of pCIB5.

Figure 7/8: Construction of pCIB4.

Figure 9: Construction of pCIB2.

Figure 10: Construction of pCIB10, a broad host range plasmid containing T-DNA borders and gene for plant selection.

Figure 11: Construction of pCIB10/19Sbt.

Figure 12: Construction of pCIB710.

Figure 13: Construction of pCIB10/710.

Figure 14: Construction of pCIB10/35Sbt.

Figure 15: Construction of pCIB10//35Sbt (KpnI).

Figure 16: Construction of pCIB10/35Sbt (BclI).

Figure 17: Construction of pCIB10/35Sbt (607).

Figure 18: not existing

Figure 19: Construction of pCIB1300, a plasmid having a chimeric gene containing the CaMV 35S promoter/AMV leader/Bt(Bcl) deletion/35S terminator.

Figures 20, 21, and 22: Construction of pCIB 1301, having a chimeric gene containing the cotton rbs-gX promoter/Bt(607 deletion) coding sequence.

Figure 23: Construction of pCIB1302, having a chimeric gene containing the cotton rbs-gY promoter/Bt(607 deletion) coding sequence.

Figure 24: Restriction map of the cotton genomic clones carrying rbc-gX and rbc-gY.

Figure 25: Nucleotide and amino acid sequences of rbc-gY. The first ATG and methionine of the transit peptide are boxed in the figure.

Figure 26: Nucleotide and amino acid sequences of rbc-gX. The first ATG and methionine of the transit peptide are boxed in the figure.

Deposits:

In connection with the present invention the following listed plasmids and/or microorganisms were deposited with the International Depository Authority "American Type Culture Collection, Rockville, Maryland" in accordance with the requirements of the Budapest Treaty.

1) *Escherichia coli* MC1061, pCIB10/35SBT....ATCC 67329
(date of deposit: February 27, 1987)

2) *Escherichia coli* HB101, pCIB10/19SBT.....ATCC 67330
(date of deposit: February 27, 1987)

3) Plasmid pLV111.....ATCC 40235
(date of deposit: May 14, 1986)

4) Phage λ/rbc-gY.....ATCC 40486
(date of deposit, August 25, 1988)

5) Phage λ/rbc-gX.....ATCC 40487
(date of deposit, August 25, 1988)

The present invention is directed to the production of a chimeric *Bt* toxin gene. The cotton plant cells contemplated include cells from any and all cotton plants into which foreign DNA can be introduced, replicated and expressed. Some suitable examples of cotton plant species include *Gossypium hirsutum*, *Gossypium arboreum*, and *Gossypium barbadense*. The above exemplification is included herein for purpose of illustration only, and is not intended to be limiting. *Gossypium hirsutum* is

preferred, and may be of the stripper or picker types. Stripper and picker cotton differ in their method of harvest, the stripper cotton bols being very firmly attached to the plant so that they are not released during late-season storms. Harvesting stripper cotton virtually destroys the plant. Picker cotton is less firmly attached and is harvested by less disruptive means. Some commercially available varieties of *G. hirsutum* capable of being regenerated by the method of the present invention include:

Acala 1515-75, Acala SJ-2, Acala SJ-4, Acala SJ-5, Acala SJC-1, Acala SJC-22, Acala SJC-28, Acala SJC-30, Acala B-1644, Acala B-1810, Acala B-2724, Acala GC-510.

Coker 304, Coker 315, Coker 201, Coker 310, Coker 312,

DP 41, DP 90,

DPL 50, DPL 20, DPL 120, DPL 775,

Lankart 611, Lankart 57,

Paymaster 145, Paymaster HS 26,

Stoneville 506, Stoneville 825,

Funk 519-2, Funk FC 3008, Funk FC 3024, Funk C 1568R, Funk FC 2005, Funk C 0947B, Funk FC 2028, Funk FC 2017, Funk C 1379,

McNair 235, Tomcot SP 21-S, Siokra, Tx-CAB-CS.

The preferred varieties are Acala SJ-2, Acala SJC-1, Acala GC 510, Acala SJC-28, Acala SJC-30, Acala B-1644 and Siokra.

Acala SJ-2, Acala GC 510, Acala B-1644, and Siokra are especially preferred.

The term "plant cell" refers to any cell derived from a cotton plant. Some examples of cells encompassed by the present invention include differentiated cells that are part of a living plant; undifferentiated cells in culture; the cells of undifferentiated tissue such as callus or tumors; seeds; embryos; propagules and pollen.

The chimeric gene of this invention contains a promoter region that functions efficiently in cotton plants and a coding region that codes for the crystal protein from *Bt* or for a polypeptide having substantially the insecticidal properties of the crystal protein from *Bt*. The coding sequence of the chimeric gene is not known to be associated with the promoter in natural genes.

The 5' and/or 3' untranslated regions may, independently, be associated in nature with either the promoter or the coding region, or with neither the promoter nor the coding region. Preferably, either the 5' or the 3' untranslated region is associated with the promoter in natural genes, and most preferably both the 5' and 3' regions are associated with the promoter in natural genes.

One could not predict, based on the state of the art at the time this invention was made, that a chimeric gene could be stably and functionally introduced into cotton cells. It was even less predictable that such cells would express an insecticidal polypeptide at any level, and especially at sufficient levels to impart insecticidal properties to the cells. In particular, a polypeptide as large and as insoluble as the polypeptide having the insect toxicity properties of *Bt* crystal protein was expected to be especially difficult to express in plant cells.

In order to be considered insecticidal, the plant cells must contain an insecticidal amount of toxin having substantially the insecticidal activity of the crystal protein from *Bt*. An insecticidal amount is an amount which, when present in plant cells, kills insect larvae or at least reduces their feeding substantially.

Accordingly, the plant cells of the present invention are able to withstand attacks by insect larvae without, or with less, application of crystal protein or other insecticides when compared with plant cells that do not contain a gene producing an insecticidal polypeptide.

The chimeric gene of this invention contains transcription control sequences comprising promoter and 5' and 3' untranslated sequences that are functional in cotton plants. These sequences may, independently, be derived from any source, such as, virus, plant or bacterial genes.

The virus promoters and 5' and 3' untranslated sequences suitable for use are functional in cotton plants and are obtained, for example, from plant viruses such as Cauliflower mosaic virus (CaMV). CaMV has been characterized and described by Hohn et al. (1982) 194-220 and appendices A to G. This description is incorporated herein by reference.

CaMV is an atypical plant virus in that it contains double-stranded DNA. At least two CaMV promoters are functional in plants, namely the 19S promoter, which results in transcription of gene VI of CaMV, and the promoter of the 35S transcript. The 19S promoter and the 35S promoter are the preferred plant virus promoters for use in the present invention.

CaMV 19S promoters and 5' untranslated regions may be obtained by means of a restriction map such as the map described in Figure 4 on page 199 of the Hohn et al. article mentioned above, or from the sequence that appears in Appendix C of the Hohn et al. article.

In order to isolate the CaMV 19S promoter and, optionally, the adjacent 5' untranslated region, a restriction fragment of the CaMV genome containing the desired sequences is selected. A suitable restriction fragment that contains the 19S promoter and the 5' untranslated region is the fragment between the PstI site starting at position 5386 and the HindIII site starting at position 5850 of Figure 4 and appendix C of the Hohn et al. article.

By analogous methods, the 35S promoter from CaMV may be obtained, as is described below.

Undesired nucleotides in the restriction fragment may optionally be removed by standard methods. Some suitable methods for deleting undesired nucleotides include the use of exonucleases (Maniatis et al., 1982) and oligonucleotide-directed mutagenesis (Zoller and Smith, 1983).

A similar procedure may be used to obtain a desirable 3' untranslated region. For example, a suitable CaMV 19S gene 3' untranslated sequence may be obtained by isolating the region between the EcoRV site at position 7342 and the BglII site at position 7643 of the CaMV genome as described in Figure 4 and appendix C of the Hohn et al. article.

Examples of plant gene promoters and 5' and 3' untranslated regions suitable for use in the present invention also include those of the gene coding for the small subunit of ribulose-1,5-bisphosphate carboxylase and chlorophyll a/b-binding protein. These plant gene regions may be isolated from plant cells in ways comparable to those described above for isolating the corresponding regions from CaMV (see Morelli et al., 1985).

Suitable promoters and 5' and 3' untranslated regions from bacterial genes include those present in the T-DNA region of *Agrobacterium* plasmids. Some examples of suitable *Agrobacterium* plasmids include the Ti plasmid of *A. tumefaciens* and the Ri plasmid of *A. rhizogenes*. The *Agrobacterium* promoters and 5' and 3' untranslated regions useful in the present invention are, in particular, those present in the genes coding for octopine synthase and nopaline synthase. These sequences may be obtained by methods similar to those described above for isolating CaMV and plant promoter and untranslated sequences (see Bevan et al., 1983).

The coding region of the chimeric gene contains a nuclectide sequence that codes for a polypeptide having substantially the toxicity properties of a *Bt* δ-endotoxin crystal protein. A polypeptide, for the purpose of the present invention, has substantially the toxicity properties of *Bt* δ-endotoxin crystal protein if it is insecticidal to a similar range of insect larvae as is the crystal protein from a subspecies of *Bt*. Some suitable subspecies include, for example, *Bt* var. *kurstaki*, *Bt* var. *berliner*, *Bt* var. *alesti*, *Bt* var. *tolworthi*, *Bt* var. *sotto*, *Bt* var.

dendrolimus; *Bt* var. *tenebrionis*; *Bt* var. *san diego*; and *Bt* var. *aizawai*. The preferred subspecies is *Bt* var. *kurstaki*, and especially *Bt* var. *kurstaki* HD1.

The coding region may exist naturally in *Bt*. Alternatively, the coding region may contain a sequence that is different from the sequence that exists in *Bt*, but is equivalent because of the degeneracy of the genetic code.

The coding sequence of the chimeric gene may also code for a polypeptide that differs from a naturally occuring crystal protein δ-endotoxin but that still has substantially the insect toxicity properties of the crystal protein. Such a coding sequence will usually be a variant of a natural coding region. A "variant" of a natural DNA sequence is a modified form of the natural sequence that performs the same function. The variant may be a mutation, or may be a synthetic DNA sequence, and is substantially homologous to the corresponding natural sequence.

"Substantial sequence homology" should be understood as referring to either: a DNA fragment having a nucleotide sequence sufficiently similar to another DNA fragment to produce a protein having similar properties; or a polypeptide having an amino acid sequence sufficiently similar to another polypeptide to exhibit similar properties.

Normally, a DNA sequence is substantially homologous to a second DNA sequence if at least 70 %, preferably at least 80 %, and most preferably at least 90 % of the active portions of the DNA sequence are homologous. Two different nucleotides are considered to be homologous in a DNA sequence of a coding region for the purpose of determining substantial homology if the substitution of one for the other constitutes a silent mutation.

The invention thus includes cotton cells and plants containing any chimeric gene coding for a sequence of amino acids having the insecticidal properties satisfying the requirements disclosed and

claimed. It is preferred that the nucleotide sequence is substantially homologous at least to that portion or to those portions of the natural sequence responsible for insecticidal activity.

The polypeptide expressed by the chimeric gene of this invention will generally also share at least some immunological properties with a natural *Bt* crystal protein, since it has at least some of the same antigenic determinants.

Accordingly, the polypeptide coded for by the chimeric gene of the present invention is preferably structurally related to the crystal δ-endotoxin protein produced by *Bt*. *Bt* produces a crystal protein with a subunit which is a protoxin having an Mr of about 130,000 to 140,000. This protoxin can be cleaved by proteases or by alkali to form insecticidal fragments having an Mr as low as 80,000, preferably about 70 000, more preferably about 60,000 and possibly even lower. The fragments preferably have a maximum Mr of about 120,000, more preferably about 110,000 and most preferably about 100,000. Chimeric genes that code for such fragments of the protoxin or for even smaller portions thereof according to the present invention can be constructed as long as the fragments or portions of fragments have the requisite insecticidal activity. The protoxin, insecticidal fragments of the protoxin and insecticidal portions of these fragments can be fused to other molecules such as polypeptides and proteins.

Coding regions suitable for use in the present invention may be obtained from crystal protein toxin genes isolated from *Bt* (for example, see PCT application WO 86/01536 and U.S. patents 4,448,885 and 4,467,036). A preferred sequence of nucleotides that codes for a crystal protein is that shown as nucleotides 156 to 3623 in the sequence of the formula I or a shorter sequence that codes for an insecticidal fragment of such a crystal protein. The disclosure of this sequence in Geiser et al. (1986) is incorporated herein by reference.

Formula I

```
        10          20          30          40          50          60
GTTAACACCC TGGGTCAAAA ATTGATATTT AGTAAAATTA GTTGCACTTT GTGCATTTTT

        70          80          90         100         110         120
TCATAAGATG AGTCATATGT TTTAAATTGT AGTAATGAAA AACAGTATTA TATCATAATG

       130         140         150         160         170         180
AATTGGTATC TTAATAAAAG AGATGGAGGT AACTTATGGA TAACAATCCG AACATCAATG

       190         200         210         220         230         240
AATGCATTCC TTATAATTGT TTAAGTAACC CTGAAGTAGA AGTATTAGGT GGAGAAAGAA

       250         260         270         280         290         300
TAGAAACTGG TTACACCCCA ATCGATATTT CCTTGTCGCT AACGCAATTT CTTTTGAGTG

       310         320         330         340         350         360
AATTTGTTCC CGGTGCTGGA TTTGTGTTAG GACTAGTTGA TATAATATGG GGAATTTTTG

       370         380         390         400         410         420
GTCCCTCTCA ATGGGACGCA TTTCTTGTAC AAATTGAACA GTTAATTAAC CAAAGAATAG

       430         440         450         460         470         480
AAGAATTCGC TAGGAACCAA GCCATTTCTA GATTAGAAGG ACTAAGCAAT CTTTATCAAA

       490         500         510         520         530         540
TTTACGCAGA ATCTTTTAGA GAGTGGGAAG CAGATCCTAC TAATCCAGCA TTAAGAGAAG

       550         560         570         580         590         600
AGATGCGTAT TCAATTCAAT GACATGAACA GTGCCCTTAC AACCGCTATT CCTCTTTTTG

       610         620         630         640         650         660
CAGTTCAAAA TTATCAAGTT CCTCTTTTAT CAGTATATGT TCAAGCTGCA AATTTACATT

       670         680         690         700         710         720
TATCAGTTTT GAGAGATGTT TCAGTGTTTG GACAAAGGTG GGGATTTGAT GCCGCGACTA

       730         740         750         760         770         780
TCAATAGTCG TTATAATGAT TTAACTAGGC TTATTGGCAA CTATACAGAT CATGCTGTAC

       790         800         810         820         830         840
GCTGGTACAA TACGGGATTA GAGCGTGTAT GGGGACCGGA TTCTAGAGAT TGGATAAGAT

       850         860         870         880         890         900
ATAATCAATT TAGAAGAGAA TTAACACTAA CTGTATTAGA TATCGTTTCT CTATTTCCGA

       910         920         930         940         950         960
ACTATGATAG TAGAACGTAT CCAATTCGAA CAGTTTCCCA ATTAACAAGA GAAATTTATA

       970         980         990        1000        1010        1020
CAAACCCAGT ATTAGAAAAT TTTGATGGTA GTTTTCGAGG CTCGGCTCAG GGCATAGAAG

      1030        1040        1050        1060        1070        1080
GAAGTATTAG GAGTCCACAT TTGATGGATA TACTTAACAG TATAACCATC TATACGGATG
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      1090        1100        1110        1120        1130        1140
CTCATAGAGG AGAATATTAT TGGTCAGGGC ATCAAATAAT GGCTTCTCCT GTAGGGTTTT

      1150        1160        1170        1180        1190        1200
CGGGGCCAGA ATTCACTTTT CCGCTATATG GAACTATGGG AAATGCAGCT CCACAACAAC

      1210        1220        1230        1240        1250        1260
GTATTGTTGC TCAACTAGGT CAGGGCGTGT ATAGAACATT ATCGTCCACT TTATATAGAA

      1270        1280        1290        1300        1310        1320
GACCTTTTAA TATAGGGATA AATAATCAAC AACTATCTGT TCTTGACGGG ACAGAATTTG

      1330        1340        1350        1360        1370        1380
CTTATGGAAC CTCCTCAAAT TTGCCATCCG CTGTATACAG AAAAAGCGGA ACGGTAGATT

      1390        1400        1410        1420        1430        1440
CGCTGGATGA AATACCGCCA CAGAATAACA ACGTGCCACC TAGGCAAGGA TTTAGTCATC

      1450        1460        1470        1480        1490        1500
GATTAAGCCA TGTTTCAATG TTTCGTTCAG GCTTTAGTAA TAGTAGTGTA AGTATAATAA

      1510        1520        1530        1540        1550        1560
GAGCTCCTAT GTTCTCTTGG ATACATCGTA GTGCTGAATT TAATAATATA ATTCCTTCAT

      1570        1580        1590        1600        1610        1620
CACAAATTAC ACAAATACCT TTAACAAAAT CTACTAATCT TGGCTCTGGA ACTTCTGTCG

      1630        1640        1650        1660        1670        1680
TTAAAGGACC AGGATTTACA GGAGGAGATA TTCTTCGAAG AACTTCACCT GGCCAGATTT

      1690        1700        1710        1720        1730        1740
CAACCTTAAG AGTAAATATT ACTGCACCAT TATCACAAAG ATATCGGGTA AGAATTCGCT

      1750        1760        1770        1780        1790        1800
ACGCTTCTAC CACAAATTTA CAATTCCATA CATCAATTGA CGGAAGACCT ATTAATCAGG

      1810        1820        1830        1840        1850        1860
GGAATTTTTC AGCAACTATG AGTAGTGGGA GTAATTTACA GTCCGGAAGC TTTAGGACTG

      1870        1880        1890        1900        1910        1920
TAGGTTTTAC TACTCCGTTT AACTTTTCAA ATGGATCAAG TGTATTTACG TTAAGTGCTC

      1930        1940        1950        1960        1970        1980
ATGTCTTCAA TTCAGGCAAT GAAGTTTATA TAGATCGAAT TGAATTTGTT CCGGCAGAAG

      1990        2000        2010        2020        2030        2040
TAACCTTTGA GGCAGAATAT GATTTAGAAA GAGCACAAAA GGCGGTGAAT GAGCTGTTTA

      2050        2060        2070        2080        2090        2100
CTTCTTCCAA TCAAATCGGG TTAAAAACAG ATGTGACGGA TTATCATATT GATCAAGTAT

      2110        2120        2130        2140        2150        2160
CCAATTTAGT TGAGTGTTTA TCTGATGAAT TTTGTCTGGA TGAAAAAAAA GAATTGTCCG
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      2170        2180        2190        2200        2210        2220
AGAAAGTCAA ACATGCGAAG CGACTTAGTG ATGAGCGGAA TTTACTTCAA GATCCAAACT

      2230        2240        2250        2260        2270        2280
TTAGAGGGAT CAATAGACAA CTAGACCGTG GCTGGAGAGG AAGTACGGAT ATTACCATCC

      2290        2300        2310        2320        2330        2340
AAGGAGGCGA TGACGTATTC AAAGAGAATT ACGTTACGCT ATTGGGTACC TTTGATGAGT

      2350        2360        2370        2380        2390        2400
GCTATCCAAC GTATTTATAT CAAAAAATAG ATGAGTCGAA ATTAAAAGCC TATACCCGTT

      2410        2420        2430        2440        2450        2460
ACCAATTAAG AGGGTATATC GAAGATAGTC AAGACTTAGA AATCTATTTA ATTCGCTACA

      2470        2480        2490        2500        2510        2520
ATGCCAAACA CGAAACAGTA AATGTGCCAG GTACGGGTTC CTTATGGCCG CTTTCAGCCC

      2530        2540        2550        2560        2570        2580
CAAGTCCAAT CGGAAAATGT GCCCATCATT CCCATCATTT CTCCTTGGAC ATTGATGTTG

      2590        2600        2610        2620        2630        2640
GATGTACAGA CTTAAATGAG GACTTAGGTG TATGGGTGAT ATTCAAGATT AAGACGCAAG

      2650        2660        2670        2680        2690        2700
ATGGCCATGC AAGACTAGGA AATCTAGAAT TTCTCGAAGA GAAACCATTA GTAGGAGAAG

      2710        2720        2730        2740        2750        2760
CACTAGCTCG TGTGAAAAGA GCGGAGAAAA AATGGAGAGA CAAACGTGAA AAATTGGAAT

      2770        2780        2790        2800        2810        2820
GGGAAACAAA TATTGTTTAT AAAGAGGCAA AAGAATCTGT AGATGCTTTA TTTGTAAACT

      2830        2840        2850        2860        2870        2880
CTCAATATGA TAGATTACAA GCGGATACCA ACATCGCGAT GATTCATGCG GCAGATAAAC

      2890        2900        2910        2920        2930        2940
GCGTTCATAG CATTCGAGAA GCTTATCTGC CTGAGCTGTC TGTGATTCCG GGTGTCAATG

      2950        2960        2970        2980        2990        3000
CGGCTATTTT TGAAGAATTA GAAGGGCGTA TTTTCACTGC ATTCTCCCTA TATGATGCGA

      3010        3020        3030        3040        3050        3060
GAAATGTCAT TAAAAATGGT GATTTTAATA ATGGCTTATC CTGCTGGAAC GTGAAAGGGC

      3070        3080        3090        3100        3110        3120
ATGTAGATGT AGAAGAACAA AACAACCACC GTTCGGTCCT TGTTGTTCCG GAATGGGAAG

      3130        3140        3150        3160        3170        3180
CAGAAGTGTC ACAAGAAGTT CGTGTCTGTC CGGGTCGTGG CTATATCCTT CGTGTCACAG

      3190        3200        3210        3220        3230        3240
CGTACAAGGA GGGATATGGA GAAGGTTGCG TAACCATTCA TGAGATCGAG AACAATACAG

      3250        3260        3270        3280        3290        3300
ACGAACTGAA GTTTAGCAAC TGTGTAGAAG AGGAAGTATA TCCAAACAAC ACGGTAACGT
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      3310        3320        3330        3340        3350        3360
GTAATGATTA TACTGCGACT CAAGAAGAAT ATGAGGGTAC GTACACTTCT CGTAATCGAG

      3370        3380        3390        3400        3410        3420
GATATGACGG AGCCTATGAA AGCAATTCTT CTGTACCAGC TGATTATGCA TCAGCCTATG

      3430        3440        3450        3460        3470        3480
AAGAAAAAGC ATATACAGAT GGACGAAGAG ACAATCCTTG TGAATCTAAC AGAGGATATG

      3490        3500        3510        3520        3530        3540
GGGATTACAC ACCACTACCA GCTGGCTATG TGACAAAAGA ATTAGAGTAC TTCCCAGAAA

      3550        3560        3570        3580        3590        3600
CCGATAAGGT ATGGATTGAG ATCGGAGAAA CGGAAGGAAC ATTCATCGTG GACAGCGTGG

      3610        3620        3630        3640        3650        3660
AATTACTTCT TATGGAGGAA TAATATATGC TTTATAATGT AAGGTGTGCA AATAAAGAAT

      3670        3680        3690        3700        3710        3720
GATTACTGAC TTGTATTGAC AGATAAATAA GGAAATTTTT ATATGAATAA AAAACGGGCA

      3730        3740        3750        3760        3770        3780
TCACTCTTAA AAGAATGATG TCCGTTTTTT GTATGATTTA ACGAGTGATA TTTAAATGTT

      3790        3800        3810        3820        3830        3840
TTTTTTGCGA AGGCTTTACT TAACGGGGTA CCGCCACATG CCCATCAACT TAAGAATTTG

      3850        3860        3870        3880        3890        3900
CACTACCCCC AAGTGTCAAA AAACGTTATT CTTTCTAAAA AGCTAGCTAG AAAGGATGAC

      3910        3920        3930        3940        3950        3960
ATTTTTTATG AATCTTTCAA TTCAAGATGA ATTACAACTA TTTTCTGAAG AGCTGTATCG

      3970        3980        3990        4000        4010        4020
TCATTTAACC CCTTCTCTTT TGGAAGAACT CGCTAAAGAA TTAGGTTTTG TAAAAAGAAA

      4030        4040        4050        4060        4070        4080
ACGAAAGTTT TCAGGAAATG AATTAGCTAC CATATGTATC TGGGGCAGTC AACGTACAGC

      4090        4100        4110        4120        4130        4140
GAGTGATTCT CTCGTTCGAC TATGCAGTCA ATTACACGCC GCCACAGCAC TCTTATGAGT

      4150        4160        4170        4180        4190        4200
CCAGAAGGAC TCAATAAACG CTTTGATAAA AAAGCGGTTG AATTTTTGAA ATATATTTTT

      4210        4220        4230        4240        4250        4260
TCTGCATTAT GGAAAAGTAA ACTTTGTAAA ACATCAGCCA TTTCAAGTGC AGCACTCACG

      4270        4280        4290        4300        4310        4320
TATTTTCAAC GAATCCGTAT TTTAGATGCG ACGATTTTCC AAGTACCGAA ACATTTAGCA

      4330        4340        4350        4360
CATGTATATC CTGGGTCAGG TGGTTGTGCA CAAACTGCAG
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The coding region defined by nucleotides 156 to 3623 of the sequence (I) encodes the polypeptide of the sequence of the formula (II).

SEQUENCE OF THE FORMULA (II)

Met Asp Asn Asn Pro Asn Ile Asn Glu Cys	10
Ile Pro Tyr Asn Cys Leu Ser Asn Pro Glu	20
Val Glu Val Leu Gly Gly Glu Arg Ile Glu	30
Thr Gly Tyr Thr Pro Ile Asp Ile Ser Leu	40
Ser Leu Thr Gln Phe Leu Leu Ser Glu Phe	50
Val Pro Gly Ala Gly Phe Val Leu Gly Leu	60
Val Asp Ile Ile Trp Gly Ile Phe Gly Pro	70
Ser Gln Trp Asp Ala Phe Leu Val Gln Ile	80
Glu Gln Leu Ile Asn Gln Arg Ile Glu Glu	90
Phe Ala Arg Asn Gln Ala Ile Ser Arg Leu	100
Glu Gly Leu Ser Asn Leu Tyr Gln Ile Tyr	110
Ala Glu Ser Phe Arg Glu Trp Glu Ala Asp	120
Pro Thr Asn Pro Ala Leu Arg Glu Glu Met	130
Arg Ile Gln Phe Asn Asp Met Asn Ser Ala	140
Leu Thr Thr Ala Ile Pro Leu Phe Ala Val	150
Gln Asn Tyr Gln Val Pro Leu Leu Ser Val	160
Tyr Val Gln Ala Ala Asn Leu His Leu Ser	170
Val Leu Arg Asp Val Ser Val Phe Gly Gln	180
Arg Trp Gly Phe Asp Ala Ala Thr Ile Asn	190
Ser Arg Tyr Asn Asp Leu Thr Arg Leu Ile	200
Gly Asn Tyr Thr Asp His Ala Val Arg Trp	210
Tyr Asn Thr Gly Leu Glu Arg Val Trp Gly	220
Pro Asp Ser Arg Asp Trp Ile Arg Tyr Asn	230
Gln Phe Arg Arg Glu Leu Thr Leu Thr Val	240
Leu Asp Ile Val Ser Leu Phe Pro Asn Tyr	250
Asp Ser Arg Thr Tyr Pro Ile Arg Thr Val	260
Ser Gln Leu Thr Arg Glu Ile Tyr Thr Asn	270
Pro Val Leu Glu Asn Phe Asp Gly Ser Phe	280
Arg Gly Ser Ala Gln Gly Ile Glu Gly Ser	290
Ile Arg Ser Pro His Leu Met Asp Ile Leu	300
Asn Ser Ile Thr Ile Tyr Thr Asp Ala His	310
Arg Gly Glu Tyr Tyr Trp Ser Gly His Gln	320
Ile Met Ala Ser Pro Val Gly Phe Ser Gly	330
Pro Glu Phe Thr Phe Pro Leu Tyr Gly Thr	340
Met Gly Asn Ala Ala Pro Gln Gln Arg Ile	350
Val Ala Gln Leu Gly Gln Gly Val Tyr Arg	360
Thr Leu Ser Ser Thr Leu Tyr Arg Arg Pro	370
Phe Asn Ile Gly Ile Asn Asn Gln Gln Leu	380
Ser Val Leu Asp Gly Thr Glu Phe Ala Tyr	390
Gly Thr Ser Ser Asn Leu Pro Ser Ala Val	400
Tyr Arg Lys Ser Gly Thr Val Asp Ser Leu	410
Asp Glu Ile Pro Pro Gln Asn Asn Asn Val	420
Pro Pro Arg Gln Gly Phe Ser His Arg Leu	430
Ser His Val Ser Met Phe Arg Ser Gly Phe	440
Ser Asn Ser Ser Val Ser Ile Ile Arg Ala	450
Pro Met Phe Ser Trp Ile His Arg Ser Ala	460
Glu Phe Asn Asn Ile Ile Pro Ser Ser Gln	470
Ile Thr Gln Ile Pro Leu Thr Lys Ser Thr	480
Asn Leu Gly Ser Gly Thr Ser Val Val Lys	490

Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu	500
Arg Arg Thr Ser Pro Gly Gln Ile Ser Thr	510
Leu Arg Val Asn Ile Thr Ala Pro Leu Ser	520
Gln Arg Tyr Arg Val Arg Ile Arg Tyr Ala	530
Ser Thr Thr Asn Leu Gln Phe His Thr Ser	540
Ile Asp Gly Arg Pro Ile Asn Gln Gly Asn	550
Phe Ser Ala Thr Met Ser Ser Gly Ser Asn	560
Leu Gln Ser Gly Ser Phe Arg Thr Val Gly	570
Phe Thr Thr Pro Phe Asn Phe Ser Asn Gly	580
Ser Ser Val Phe Thr Leu Ser Ala His Val	590
Phe Asn Ser Gly Asn Glu Val Tyr Ile Asp	600
Arg Ile Glu Phe Val Pro Ala Glu Val Thr	610
Phe Glu Ala Glu Tyr Asp Leu Glu Arg Ala	620
Gln Lys Ala Val Asn Glu Leu Phe Thr Ser	630
Ser Asn Gln Ile Gly Leu Lys Thr Asp Val	640
Thr Asp Tyr His Ile Asp Gln Val Ser Asn	650
Leu Val Glu Cys Leu Ser Asp Glu Phe Cys	660
Leu Asp Glu Lys Lys Glu Leu Ser Glu Lys	670
Val Lys His Ala Lys Arg Leu Ser Asp Glu	680
Arg Asn Leu Leu Gln Asp Pro Asn Phe Arg	690
Gly Ile Asn Arg Gln Leu Asp Arg Gly Trp	700
Arg Gly Ser Thr Asp Ile Thr Ile Gln Gly	710
Gly Asp Asp Val Phe Lys Glu Asn Tyr Val	720
Thr Leu Leu Gly Thr Phe Asp Glu Cys Tyr	730
Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu	740
Ser Lys Leu Lys Ala Tyr Thr Arg Tyr Gln	750
Leu Arg Gly Tyr Ile Glu Asp Ser Gln Asp	760
Leu Glu Ile Tyr Leu Ile Arg Tyr Asn Ala	770
Lys His Glu Thr Val Asn Val Pro Gly Thr	780
Gly Ser Leu Trp Pro Leu Ser Ala Pro Ser	790
Pro Ile Gly Lys Cys Ala His His Ser His	800
His Phe Ser Leu Asp Ile Asp Val Gly Cys	810
Thr Asp Leu Asn Glu Asp Leu Gly Val Trp	820
Val Ile Phe Lys Ile Lys Thr Gln Asp Gly	830
His Ala Arg Leu Gly Asn Leu Glu Phe Leu	840
Glu Glu Lys Pro Leu Val Gly Glu Ala Leu	850
Ala Arg Val Lys Arg Ala Glu Lys Lys Trp	860
Arg Asp Lys Arg Glu Lys Leu Glu Trp Glu	870
Thr Asn Ile Val Tyr Lys Glu Ala Lys Glu	880
Ser Val Asp Ala Leu Phe Val Asn Ser Gln	890
Tyr Asp Arg Leu Gln Ala Asp Thr Asn Ile	900
Ala Met Ile His Ala Ala Asp Lys Arg Val	910
His Ser Ile Arg Glu Ala Tyr Leu Pro Glu	920
Leu Ser Val Ile Pro Gly Val Asn Ala Ala	930
Ile Phe Glu Glu Leu Glu Gly Arg Ile Phe	940
Thr Ala Phe Ser Leu Tyr Asp Ala Arg Asn	950
Val Ile Lys Asn Gly Asp Phe Asn Asn Gly	960
Leu Ser Cys Trp Asn Val Lys Gly His Val	970
Asp Val Glu Glu Gln Asn Asn His Arg Ser	980
Val Leu Val Val Pro Glu Trp Glu Ala Glu	990
Val Ser Gln Glu Val Arg Val Cys Pro Gly	1000
Arg Gly Tyr Ile Leu Arg Val Thr Ala Tyr	1010
Lys Glu Gly Tyr Gly Glu Gly Cys Val Thr	1020
Ile His Glu Ile Glu Asn Asn Thr Asp Glu	1030
Leu Lys Phe Ser Asn Cys Val Glu Glu Glu	1040
Val Tyr Pro Asn Asn Thr Val Thr Cys Asn	1050

Asp	Tyr	Thr	Ala	Thr	Gln	Glu	Glu	Tyr	Glu	1060
Gly	Thr	Tyr	Thr	Ser	Arg	Asn	Arg	Gly	Tyr	1070
Asp	Gly	Ala	Tyr	Glu	Ser	Asn	Ser	Ser	Val	1080
Pro	Ala	Asp	Tyr	Ala	Ser	Ala	Tyr	Glu	Glu	1090
Lys	Ala	Tyr	Thr	Asp	Gly	Arg	Arg	Asp	Asn	1100
Pro	Cys	Glu	Ser	Asn	Arg	Gly	Tyr	Gly	Asp	1110
Tyr	Thr	Pro	Leu	Pro	Ala	Gly	Tyr	Val	Thr	1120
Lys	Glu	Leu	Glu	Tyr	Phe	Pro	Glu	Thr	Asp	1130
Lys	Val	Trp	Ile	Glu	Ile	Gly	Glu	Thr	Glu	1140
Gly	Thr	Phe	Ile	Val	Asp	Ser	Val	Glu	Leu	1150
Leu	Leu	Met	Glu	Glu	End					1156

Hence, the present invention is further directed to the polypeptide having the sequence of the formula (II) or to insecticidal parts of this polypeptide.

Furthermore, it has been shown that the toxins of some *Bt* strains are toxic to other than lepidopteran insects. Specifically the toxin of *Bt* var. *tenebrionis* is, for example, toxic to coleopteran insects. The toxicity of *Bt* strain *san diego* toward coleopteran insects and the sequence of the associated toxin gene is disclosed in EP-0,202,739 and EP-0,213,818.

In order to introduce the chimeric gene of the present invention into plant cells, the gene is first inserted into a vector. If the gene is not available in an amount sufficient for transformation, the vector may be amplified by replication in a host cell. The most convenient host cells for amplification are bacterial or yeast cells. When a sufficient amount of the chimeric gene is available, it is introduced into cotton cells or tissue. The introduction of the gene into cotton plant cells or tissue may be by means of the same vector used for replication, or by means of a different vector.

Some examples of bacterial host cells suitable for replicating the chimeric gene include those selected from the group consisting of the genera *Escherichia* such as *E. coli* and *Agrobacterium* such as *A. tumefaciens* or *A. rhizogenes*. Methods for cloning heterologous genes in bacteria are described in U.S. patents 4,237,224 and 4,468,464.

The replication of genes coding for the crystal protein of *Bt* in *E. coli* is described in Wong et al. (1983).

The preferred bacterium host cell for amplifying the chimeric Bt genes of this invention is *Agrobacterium*. The advantage of amplifying the gene in *Agrobacterium* is that the *Agrobacterium* may then be used to insert the amplified gene into plant cells without further genetic manipulation.

Some examples of yeast host cells suitable for replicating the genes of this invention include those of the genus *Saccharomyces*.

Any vector into which the chimeric gene can be inserted and which replicates in a suitable host cell, such as in bacteria or yeast, may be used to amplify the genes of this invention. The vector may, for example, be derived from a phage or a plasmid. Some examples of vectors derived from phages useful in the invention include those derived from M13 and from λ. Some suitable vectors derived from M13 include M13mp18 and M13mp19. Some suitable vectors derived from λ include λgt11, λgt7 and λCharon4.

Some vectors derived from plasmids especially suitable for replication in bacteria include pBR322 (Bolivar et al., 1977); pUC18 and pUC19 (Norrander et al., 1983); and Ti plasmids (Bevan et al., 1983). The preferred vectors for amplifying the genes in bacteria are pBR322, pUC18 and pUC19.

In order to construct a chimeric gene suitable for replication in bacteria, a promoter sequence, a 5' untranslated sequence, a coding sequence and a 3' untranslated sequence are inserted into or are assembled in the proper order in a suitable vector, such as a vector described above. In order to be suitable, the vector must be able to replicate in the host cell.

The promoter, 5' untranslated region, coding region and 3' untranslated region, which comprise the chimeric gene of the invention, may first be combined in one unit outside the vector, and then inserted into the vector. Alternatively, portions of the chimeric gene may be inserted into the vector separately. The vector preferably also contains a gene that confers a trait on the host cell permitting the selection of cells

containing the vector. A preferred trait is antibiotic resistance. Some examples of useful antibiotics include ampicillin, tetracycline, hygromycin, G418, chloramphenicol, kanamycin and neomycin.

Insertion or assembly of the gene in the vector is accomplished by standard methods such as the use of recombinant DNA (Maniatis et al., 1982) and homologous recombination (Hinnen et al., 1978).

Using known recombinant DNA methods, the vector is cut, the desired DNA sequence is inserted between the cut pieces of the vector, and the ends of the desired DNA sequence are ligated to the corresponding ends of the vector.

The vector is most conveniently cut by means of suitable restriction endonucleases. Some suitable restriction endonucleases include those which form blunt ends, such as SmaI, HpaI and EcoRV, and those which form cohesive ends, such as EcoRI, SacI and BamHI.

The desired DNA sequence normally exists as part of a larger DNA molecule such as a chromosome, plasmid, transposon or phage. The desired DNA sequence is excised from its source, and optionally modified so that the ends can be joined to the ends of the cut vector. If the ends of the desired DNA sequence and of the cut vector are blunt ends, they are joined by blunt end ligases such as T4 DNA ligase.

The ends of the desired DNA sequence may also be joined to the ends of the cut vector in the form of cohesive ends, in which case a cohesive end ligase, which may also be T4 DNA ligase is used. Other suitable cohesive end ligases include, for example, *E. coli* DNA ligase.

Cohesive ends are most conveniently formed by cutting the desired DNA sequence and the vector with the same restriction endonuclease. In such a case, the desired DNA sequence and the cut vector have cohesive ends that are complementary to each other.

The cohesive ends may also be constructed by adding complementary homopolymer tails to the ends of the desired DNA sequence and to the cut vector using terminal deoxynucleotidyl transferase, or by adding a synthetic oligonucleotide sequence recognized by a particular restriction endonuclease, known as a linker, and cleaving the sequence with the endonuclease (see, for example, Maniatis et al., 1982).

The *Bt* toxin genes of the present invention may be introduced directly into plant cells by taking advantage of certain plasmids present in *Agrobacterium*. These plasmids contain regions that are naturally inserted into the genome of plant cells infected by *Agrobacterium*. The inserted region is called T-DNA (transferred-DNA). These plasmids, examples of which include the Ti (tumor inducing) plasmid of *A. tumefaciens* and the Ri (root inducing) plasmid of *A. rhizogenes*, contain T-DNA border sequences, at least one of which is believed to be necessary for the transfer of the T-DNA region from the plasmid to the genome of the infected plant cell. Natural Ti and Ri plasmids also contain virulence regions, the location of which is believed to be outside of the T-DNA region. The virulence regions are needed for the transfer of T-DNA to plant cells.

In modified systems the virulence regions may exist on plasmids different from the plasmids that contain the T-DNA. Such virulence region-containing plasmids are called helper plasmids.

The T-DNA regions that occur naturally are oncogenic and cause plant tumors. The oncogenic portions of these T-DNA regions may be partially or fully removed before, or simultaneously with, the insertion of the desired DNA sequence. The plasmids containing such modified T-DNA regions are said to be disarmed.

The genes suitable for use in the present invention are assembled in or are inserted into a T-DNA vector system by methods known in the art (Barton and Chilton, 1983; Chilton, 1985). The T-DNA vector may be oncogenic (Hernalsteens et al., 1980), partially disarmed (Barton and Chilton, 1983), fully disarmed (Zambryski et al., 1983), or may be based on artificial T-DNA vectors having synthetic T-DNA border-like sequences,

(Wang et al., 1984). Some suitable disarmed vectors containing T-DNA border regions include pGA436, pGA437 and pGA438, as are described in An et al. (1985); pMON120 (see Fraley et al., 1983) and pCIB10 (Rothstein et al., 1987). The transfer of T-DNA is usually accomplished by incubating *Agrobacterium* with plant cell protoplasts or wounded plant tissue (see Caplan et al., 1983).

In addition to the chimeric gene coding for a *Bt* or a *Bt*-like toxin, the vectors preferably further comprise a DNA sequence that permits the selection or screening of cotton plant cells containing the vector in the presence of cells that do not contain the vector. Such selectable or screenable markers may naturally be present in the vector into which the chimeric gene of this invention is introduced, or may be introduced into the vector either before or after the chimeric gene is introduced. Alternatively, the selectable or screenable marker gene or a portion thereof may first be joined to the desired chimeric gene or any portion thereof and the recombined genes or gene segments may be introduced as a unit into the vector. The selectable or screenable marker may itself be chimeric.

The preferred selectable marker is a gene coding for antibiotic resistance. The gene must be capable of expression in the cells to be transformed. The cells can be cultured in a medium containing the antibiotic, and those cells containing the vector, which have an enhanced ability to survive in the medium, are selected. Genes that confer resistance to chloramphenicol, kanamycin, hygromycin, G418 or, in principle, any other antibiotic may be useful as a selectable marker.

Some examples of genes that confer antibiotic resistance include, for example, those coding for neomycin phosphotransferase [kanamycin and G418 resistance, Velten et al., 1984]; hygromycin phosphotransferase [hygromycin resistance, van den Elzen et al., 1985]; and chloramphenicol acetyltransferase.

An example of a gene useful primarily as a screenable marker in tissue culture for identification of plant cells containing genetically engineered vectors is a gene that encodes an enzyme having a chromogenic

substrate. For example, if the gene encodes the enzyme β-galactosidase, the plant cells are plated on a tissue culture medium containing the chromogenic substrate Xgal (5-chloro-4-bromo-3-indolyl-β-D-galactoside), and under appropriate conditions, plant cells containing copies of this gene are stained blue by the dye indigo which is released when β-galactosidase cleaves Xgal.

The introduction of chimeric genes into plants in accordance with the present invention may be carried out with any T-DNA-derived vector system capable of introducing genes into cotton plant cells from Agrobacteria. The vector system may, for example, be a co-integrate system (Comai et al., 1983; Zambryski et al., 1983) for example the split-end vector system (Fraley et al., 1985), as described by Chilton (1985). The vector system may, on the other hand, be a binary system (de Framond et al., 1983; Hoekema et al., 1983), or a Ti plasmid engineered by homogenotization of the gene into the T-DNA (Matzke and Chilton, 1981). A further possibility is a system wherein the T-DNA is on a plasmid and the virulence genes are on the chromosonal DNA.

The preferred T-DNA vector system is a binary vector system, and especially a system utilizing pCIB10 (Rothstein et al., 1987) (see figure 10).

The introduction of heterologous genes by recombinant DNA manipulation into a binary vector system is described by Klee et al., 1985. The insertion of genes into a T-DNA vector may be by homologous recombination using a double recombination strategy (Matzke and Chilton, 1981); single recombination strategy (Comai et al., 1983); Zambryski et al., 1983); or a single recombination strategy with no repeats in the T-DNA (Fraley et al., 1985) as described by Chilton (1985).

If the vectors containing the chimeric gene are not assembled in *Agrobacterium*, they may be introduced into *Agrobacterium* by methods known in the art. These methods include transformation and conjugation.

Transformation involves adding naked DNA to bacteria. *Agrobacterium* may be made susceptible to the introduction of naked DNA by freezing and thawing. The transformation of *Agrobaceterium* is described by Holsters et al. (1978).

Conjugation involves the mating of a cell containing the desired vector, usually *E. coli*, with *Agrobacterium*. This r 'hod is described by Comai et al. (1983) and Chilton et al. (1976).

The *Agrobacterium* spp. may be any strain of *Agrobacterium* capable of introducing genes into cotton plant cells. Some suitable examples include *A. tumefaciens*, *A. rhizogenes*, and *A. radiobacter*.

Transformed cotton plant cells containing the chimeric gene may be maintained in culture or may be regenerated into living plants. Expesssion is preferably of sufficient efficiency to render the plant cells insecticidal.

The medium capable of sustaining a particular plant cell in culture depends on the particular variety of cotton plant cell. For example, some suitable media include approximately 10 mg/liter of 2,4-dichlorophenoxy-acetic acid and either Murashige and Skoog inorganic salts (Murashige and Skoog, 1962) or Gamborg B-5 inorganic salts (Gamborg et al., 1968).

Cotton (*Gossypium* spp.) embryos capable of germination and regeneration can be efficiently produced through somatic embryogenesis by developing pro-embryonic cell masses and, from them, embryos in a cell suspension culture system.

The present method permits the production, for example, in a standard 250 ml DeLong flask of about 10,000 globular embryos, from which about 1000 mature embryos and about 50 plants may be obtained.

The cotton plants produced in accordance with this method may be cultivated or wild. Cultivated cotton plants are preferred.

Step a: Embryogenic Cotton Callus

The first step is to induce cotton callus formation from cotton explant tissue. Some examples of suitable cotton explant tissue include somatic embryos, mature and immature zygotic embryos, cotyledons or hypocotyls from a seedling, and young tissue from a mature plant. Somatic embryos and seedling cotyledons or hypocotyls are preferred.

Zygotic embryos, for example, may be obtained by excision from ovules. The ovules are preferably excised about 7 to 30 days after pollination, preferably about 10 to 21 days after pollination, and most preferably about 12 to 16 days after pollination.

Cotyledons and hypocotyls may be obtained from young seedlings. The seedlings are preferably between about 3 and 21 days old, more preferably between about 4 and 9 days old, and most preferably about 7 days old. Hypocotyls are sliced longitudinally and cut into convenient sections for example between 1 and 20 mm, preferably about 2 mm. Cotyledon tissue is cut into sections between 1 and 400 mm^2, preferably between 5 and 100 mm^2, and most preferably about 10 mm^2.

Somatic embryos derived from this procedure are the most preferred source for obtaining embryogenic callus according to the present method.

Somatic embryos may, for example, be obtained by using the method described above for hypocotyl and cotyledonary tissue as the explant source. Any somatic embryo taken before primary leaf expansion is suitable. The size of the somatic embryo is not critical. Preferably, the somatic embryo is less than about 5 mm in length.

Young tissue from a mature cotton plant may conveniently be obtained by excising the apical 10 cm, preferably about 5 cm, of a shoot tip. Stem and petiole tissue are sliced longitudinally and cut into the same size sections as are hypocotyls (see above). Leaf tissue is cut into the same size section as cotyledon tissue (see above).

The cotton plant tissue is placed on a suitable callus induction medium at about 20° to 40°C, preferably 23° to 35°C, more preferably about 31°C. Any medium capable of inducing callus from the tissue may be used in this regeneration method. The medium may be liquid or solid, although a solid medium is preferred since it is more convenient.

One medium capable of inducing callus under the conditions of the invention contains inorganic salts, vitamins, a carbon source, an auxin, and a cytokinin. The medium is adjusted to a pH between 3.5 and 7.5, preferably between 4.5 and 6.5, and most preferably about 5.7.

Any inorganic salts and vitamins capable of contributing to callus induction are suitable. Some examples of suitable inorganic salts and vitamins include those described by Murashige and Skoog (1962) (MS) and Gamborg et al. (1968) (B-5). Another example is a modification of MS or Gamborg's B-5 media described by Cheng et al. (1980). The preferred inorganic salts are MS inorganic salts. The preferred vitamins are Gamborg's B-5 vitamins.

The carbon source may be any carbon source on which callus can be grown. The preferred carbon sources include sugars and derivatives of sugar. The preferred sugars are glucose and sucrose. It is especially desirable to initiate callus in a callus induction medium containing glucose in order to reduce browning of the tissue, and then to transfer the callus to a callus induction medium containing sucrose.

The concentration of the carbon source is 5 to 60 g/liter, preferably about 30 g/liter.

The auxin present in the callus induction medium may be any auxin capable of inducing callus. Some suitable auxins include α-naphthaleneacetic acid, picloram, 2,4,5-trichlorophenoxyacetic acid, 2,4-dichlorophenoxy-acetic acid, indole-3-butyric acid, indole-3-lactic acid, indole-3-pyruvic acid, indole-3-acetic acid, and p-chlorophenoxyacetic acid. A preferred auxin is α-naphthaleneacetic acid.

Any concentration of auxins capable of inducing callus formation may be used in the method of the invention. A suitable concentration is 0.1 to 10 mg/liter. A preferred concentration is about 2 mg/liter, especially when the auxin is α-naphthaleneacetic acid.

The cytokinin present in the callus induction medium may be any cytokinin capable of inducing callus. Some suitable cytokinins include kinetin, 6-benzyladenine, 2-isopentenyladenine, and zeatin. A preferred cytokinin is kinetin.

Any concentration of cytokinin capable of inducing callus formation may be used in the method of the invention. Suitable concentrations are 0.1 to 10 mg/liter. A preferred concentration is 1 mg/liter, especially when the cytokinin is kinetin.

If the medium is solid, it contains a component that causes solidification, for example about 0.8 % agar such as Agar Noble (Difco) or about 0.8 % agarose. (All percents in this specification are by weight).

The tissue is cultured on the callus induction medium for a period of time sufficient for the callus to form. For example, tissue may be cultured on a callus induction medium containing glucose as the carbon source. A five week induction period is typical. Subcultures are performed as necessary to prevent browning. Weekly subcultures are preferred.

The callus that forms may be unorganized, or may contain pro-embryonic cell masses, embryogenic callus and/or embryos. Normally, when hypocotyls or cotyledons are used as the explant source, the callus appears to be unorganized. When somatic embryos are used as the explant source, at least part of the callus appears to comprise embryogenic callus, which is characterized by a light yellow color and nodulation.

The resulting callus may then advantageously be transferred to a callus subculture medium, which is similar to a callus induction medium except that it contains sucrose as the carbon source, for a period of time up to

5 months. One month, or two months with a subculture into fresh medium after one month, on a sucrose-containing callus induction medium is preferred.

The callus may be induced in the dark, but is preferably induced in the light. The light may have an intensity of, for example, 0.5 to 150 $\mu Em^{-2}s^{-1}$ (= 41.75 to 12525 lx).

Step b: Clumpy Aggregates of Pro-embryonic Cell Masses

The callus from step (a) is suspended in a liquid medium promoting the development of pro-embryonic or proliferating embryonic cell masses. It is important for the cell density to be low. Therefore, not more than 40 mg of callus/ml of culture medium, preferably not more than 15 mg of callus/ml of culture medium and more preferably not more than 5 mg of callus/ml of culture medium is suspended.

The medium useful in step (b) may be any medium capable of inducing pro-embryonic cell masses. The medium comprises inorganic salts, vitamins, a carbon source, and an auxin. The medium may also include organic nitrogen sources, cytokinins, amino acids and other addenda such as casein hydrolysate or coconut water.

The inorganic salts and vitamins may be the same as in step (a) (supra). MS inorganic salts and B-5 vitamins are preferred.

The carbon source may be the same as in step (a) (supra). Sucrose is preferred. The concentration of the carbon source is 0.1 to 100 g/liter. About 20 g/liter is preferred, especially when the carbon source is sucrose.

The auxin may be selected from the auxins used in step (a). The preferred auxins are 2,4-dichlorophenoxyacetic acid and picloram. Picloram is most preferred.

The concentration of the auxin in step (b) is relatively low. The exact concentration depends on the specific auxin used. The relatively low auxin concentration is generally similar to that usually used in sus-

pension culture media and is significantly lower than the corresponding auxin concentration used in step (c). When picloram is the auxin used in step (b), the concentration is 0.01 to 5 mg/liter, preferably 0.1 to 1 mg/liter, and most preferably about 0.5 mg/liter. When 2,4-dichlorophenoxyacetic acid is the auxin used in step (b), the concentration is 0.01 to 0.5 mg/liter, preferably 0.05 to 0.25 mg/liter, and most preferably about 0.1 mg/liter.

The induction of pro-embryonic cell masses is preferably carried out in an aerated medium at a temperature between 20° and 35°C, preferably between 22° and 33°C and most preferably between 25° and 31°C. One may aerate the medium by any method known in the art, for example by shaking. Step (b) may be carried out in the dark or in light up to about 75 $\mu Em^{-2}s^{-1}$ (= 6262,5 lx), preferably between 5 and 10 $\mu Em^{-2}s^{-1}$ (= 417,5 and 835 lx).

The callus is maintained in the medium preferably without subculture until clumpy aggregates of pro-embryonic cell masses form and begin to proliferate rapidly. The onset of rapid proliferation usually takes between 3 and 8 weeks, more typically between 5 and 7 weeks. During the induction period, the medium may be replaced by fresh medium, although it is preferable not to disturb the medium during this period.

The change from callus to clumpy aggregates of pro-embryonic cell masses will be readily apparent to those of ordinary skill in the art of plant tissue culture. It is distinguishable by the light yellow color and clumpy nature of the pro-embryonic cell masses.

Once the clumpy aggregates of pro-embryogenic cell masses begin to proliferate rapidly, they may be introduced directly into the medium described in step (c), or they may be subcultured in order to prevent browning. Subculturing every 3 to 7 days, preferably every 5 to 7 days, is convenient. The cell masses survive without subculture for about fourteen days.

Step c: Finely Dispersed Pro-embryonic Cell Masses

The clumpy aggregates of pro-embryonic cell masses from step (b) are transferred to a liquid medium that is capable of causing the clmpy aggregates of pro-embryonic cell masses to become finely dispersed. The medium may be similar to that described in step (b), except that the medium of step (c) comprises a relatively high concentration of an auxin. The auxin may be any auxin used in step (a). The preferred auxins are 2,4,5-trichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid. The most preferred auxin is 2,4-dichlorophenoxyacetic acid.

The concentration of auxin depends on the particular auxin used. The auxin concentration in the medium of step (c) is generally higher, or at least at the high end of the range of, than concentrations that are usually used in suspension culture media, and in any event is significantly higher than the corresponding auxin concentration used in step (b).

For example, when 2,4-dichlorophenoxyacetic acid is the auxin in the medium of step (c), the concentration may be about 0.5 to 100 mg/liter, preferably 1 to 10 mg/liter, and most preferably about 2.5 to 7.5 mg/liter.

Except for the concentration and possibly the identity of the auxin, the medium, temperature, and amount of light in step (c) may be the same as that described in step (b).

The conditions of step (c) are maintained until the clumpy aggregates of pro-embryonic cell masses become smaller, more finely dispersed pro-embryonic cell masses. The appearance of the smaller, more finely dispersed pro-embryonic cell masses will be readily apparent to those skilled in the art. These cell masses are characterized by their yellow color, smooth surface, intermediate density and small size. The change to the smaller, more finely dispersed cell masses usually occurs within 6 weeks, more typically 2 weeks.

The culture of the small finely dispersed pro-embryonic cell masses may be maintained indefinitely, and may be subcultured so as to maintain active growth. It is convenient to subculture, for example, every 3 to 28 days, preferably every 5 to 10 days.

Step d: Mature Embryos

The smaller, more finely dispersed pro-embryonic cell masses are added to a medium that induces the development of mature embryos. The medium is preferably a liquid.

Embryos pass through a number of developmental stages before they mature and are able to germinate. These stages include globular, heart, torpedo and mature stages. The names of the stages are based on the approximate shapes of the embryos.

The medium useful in step (d) may be any medium that induces the development of mature embryos. One useful medium comprises inorganic salts, vitamins, a carbon source and an organic compound containing reduced nitrogen.

The salts and vitamins and concentrations thereof may be the same as those described in step (a). The carbon source may also be one of the carbon sources described in step (a). The concentration of the carbon source is about 1 to 10 g/liter preferably about 2 to 6 g/liter. A preferred carbon source is sucrose.

The organic nitrogen source may be any such compound which, when added to the medium of step (d), induces the development of mature embryos. The preferred compounds are amino acids. A preferred amino acid is glutamine.

The concentration of the organic nitrogen source depends on the particular compound used. An effective concentration of glutamine as the organic nitrogen source is 2 to 260 mM, preferably 5 to 100 mM, and most preferably 10 to 50 mM.

The medium of step (d) may contain an auxin. Auxins are desirable during the early stages of embryo development, but not during the later stages. Therefore, if auxins are present at all, they are preferably present only until the heart stage of development. Then, the embryos are transferred to a medium that contains no auxin.

If present, the auxin concentration may be 0.01 to 0.1 mg/liter.

The auxin may be one of the auxins useful in step (a). The preferred auxins are picloram and 2,4-dichlorophenoxyacetic acid.

The embryos may be cultured in the medium of step (d) at temperatures of 20° to 35°C in the dark or in light. The intensity of the light may be, for example, 5 to 75 $\mu Em^{-2}s^{-1}$ (= 6262.5 lx).

The embryos are maintained in the medium of step (d) until the embryos have matured into torpedo or mature states. Those skilled in the art of plant tissue culture will be able to recognize the globular, heart, torpedo and mature embryos as they form. The embryos mature, typically, in 2 to 5 weeks, usually in 3 to 4 weeks. It is usually unnecessary to subculture the embryos or to transfer the embryos to fresh medium, except possibly to change from an auxin-containing medium to a medium not containing an auxin at the heart stage.

Step e: Germination

The mature embryos are placed on a solid medium capable of inducing germination. The medium comprises inorganic salts, vitamins, and a carbon source. The medium is solidified with a suitable solidifying agent such as Gelrite (Kelko, San Diego, California), agarose or agar.

The inorganic salts may be those described in step (a), modified so that nitrate is present at high concentration while ammonium is either absent or is present at very low concentration. The concentration of nitrate may be 20 to 60 mM, preferably 30 to 60 mM, more preferably 35 to 45 mM. The concentration of ammonium ion should be no greater than 5 mM.

The source of carbon is preferably a sugar. The preferred sugar is sucrose. The concentration of the carbon source depends on the particular carbon source used. For example, when sucrose is the carbon source, the concentration is 0.1 to 6 % by weight, preferably 0.5 to 4 %, more preferably 1 to 3 %.

An organic nitrogen source is optionally present in the medium of step (e). The organic compound is preferably an amino acid or a mixture of amino acids capable of supporting germination. Preferred amino acids or mixtures thereof are glutamine or casein hydrolysate.

The concentration of the organic nitrogen source depends on the specific compound used. For example, when the compound is glutamine, the concentration may be 2 to 50 mM, preferably 5 to 30 mM, more preferably 10 to 20 mM. When the compound is casein hydrolysate or modified casein hydrolysate, the concentration is 100 to 3000 mg/liter, preferably 1000 to 2800 mg/liter, more preferably 1500 to 2500 mg/liter.

Preferably, germination occurs on a medium containing an organic nitrogen source until shoots develop. The embryos are then transferred to a medium containing no organic nitrogen source for elongation of roots.

The density of embryos in the medium is limited to a density less than that which causes development to be self-inhibitory. Suitable densities include 1 to 100 embryos in a 9 cm petri disk containing about 10 to 75 ml of medium, preferably 25 to 50 ml of medium, and most preferably about 35 ml of medium.

The medium or media of step (e) are maintained at 20° to 30°C. Preferably, the temperature is about 25°C.

Some light is necessary in step (e). An intensity of light between 5 and 150 $\mu Em^{-2}s^{-1}$ (= 417.5 to 12525 lx), preferably between 10 and 75 $\mu Em^{-2}s^{-1}$ (= 835 to 6262.5 lx), is suitable.

The embryos are maintained on the medium or media of step (e) until the embryos have germinated, typically 1 to 20 days, usually 2 to 4 days. Those skilled in the art will know when the embryos have germinated.

Step f: Plants

Following germination, the plantlets are transferred to soil for growth into plants. The transferred plants are initially covered with a glass to maintain high humidity. After one week under glass, no special treatment of the plantlets or of the plants is necessary.

Utility - Propagation

Mature embryos may be used for mass propagation and cloning. This entails either germinating the embryos and transplanting the plantlets to soil, to other growth substrates, or other plant growth environments. Mature embryos may also be enclosed in an artificial seed coat and planted as "somatic seeds". Mass propagation and cloning is beneficial if hybrid parents or a hybrid itself needs to be mass produced.

Cells, pro-embryos, embryos, plantlets and plants may be analyzed at any time during the stages described above in order to determine whether any new trait is present as a result of genetic alteration. The trait may be a useful in vitro or in planta trait. Some examples of useful traits include phytotoxin tolerance, drought tolerance, cold tolerance, disease tolerance, etc.

The cells of steps (a), (b) and (c) may also be subjected to tissue culture methods capable of producing cells or plants having desirable properties, such as herbicide tolerance. Some examples of such methods include, for example, Chaleff and Ray (1984).

The invention therefore also includes living cotton plants, the cells of which contain the chimeric gene that encodes and expresses the polypeptide having substantially the insect toxicity properties of *Bt* crystal protein.

The plant cells of this invention contain the chimeric gene and may be used to produce the polypeptide having substantially the insect toxicity of a *Bt* crystal protein. The plant cells per se may constitute the insecticide. Plant cells used directly as insecticides may be cultured plant cells, or may be components of a living plant.

The toxin may also be isolated from the plant cells by known methods such as, for example, by extraction or chromatography. The extract may be the total plant cell extract, a partially purified extract, or a pure preparation of the polypeptide. Any such extract or chromatographic isolate may be used in the same way as crystal protein from *Bt* (see, for example, Deacon, 1983, Miller et al., 1983).

The insecticidal cells of the present invention are toxic to insects that attack cotton cells and plants.

Hence, the present invention provides a method for producing in cotton a polypeptide having substantially the insect toxicity properties of a *Bt* crystal protein, which method comprises:

(a) introducing into cotton cells a gene coding for a polypeptide having substantially the insect toxicity properties of a *Bt* crystal protein wherein the promoter, 5' untranslated region, and optionally, the 3' untranslated region of the gene are derived from a plant or plant virus gene, and

(b) expressing the polypeptide.

The present ~~invention also provides~~ application describes a method of controlling insect larvae comprising feeding the larvae an insecticidal amount of transgenic cotton cells containing a gene coding for a *Bt* crystal toxin or a polypeptide having substantially the insect toxicity properties of a *Bt* crystal protein.

The present ~~invention also includes~~ application describes a method for killing or controlling insect larvae comprising feeding the larvae an insecticidal amount of transgenic cotton plant cells that contain the chimeric gene of the invention. Furthermore, the present invention also includes a method for

killing coleopteran larvae by feeding them an insecticidal amount of cells containing the chimeric gene having the coding sequence of the *Bt* var. *tenebrionis* crystal toxin or insecticidal parts thereof.

The plant cells may be cultured plant cells, or may be components of living plants.

The present invention further includes corn seeds of plants genetically engineered in accordance with this invention as long as the seeds contain the inserted chimeric gene and the desirable trait resulting therefrom. Progeny of plants produced by the method of this invention, including sexual and vegetative progeny, are further embodiments. Sexual progeny may result from selfing or cross pollination.

Non limiting Examples

General Recombinant DNA Techniques

Since many of the recombinant DNA techniques used in this invention are routine for those skilled in the art, a brief description of these commonly used techniques is included here rather than at each instance where they appear below. Except where noted, all of these routine procedures are described in the reference by Maniatis et al. (1982).

A. Restriction endonuclease digestions

Typically, DNA is present in the reaction mixture at approximately 50-500 µg/ml in the buffer solution recommended by the manufacturer, New England Biolabs, Beverly, MA. 2 to 5 units of restriction endonucleases are added for each µg of DNA, and the reaction mixture incubated at the temperature recommended by the manufacturer for one to three hours. The reaction is terminated by heating to 65°C for ten minutes or by extraction with phenol, followed by precipitation of the DNA with ethanol. This technique is also described on pages 104-106 of the Maniatis et al. reference.

B. Treatment of DNA with polymerase to create flush ends

DNA fragments are added to a reaction mixture at 50-500 µg/ml in the buffer recommended by the manufacturer, New England Biolabs. The reaction mixture contains all four deoxynucleotide triphosphates at a concentration of 0.2 mM. The reaction is incubated at 15°C for 30 minutes, and then terminated by heating to 65°C for ten minutes. For fragments produced by digestion with restriction endonucleases that create 5'-protruding ends, such as EcoRI and BamHI, the large fragment, or Klenow fragment, of DNA polymerase is used. For fragments produced by endonucleases that produce 3'-protruding ends, such as PstI and SacI, T4 DNA polymerase is used. Use of these two enzymes is described on pages 113-121 of the Maniatis et al. reference.

C. Agarose gel electrophoresis and purification of DNA fragments from gels

Agarose gel electrophoresis is performed in a horizontal apparatus as described on pages 150-163 of Maniatis et al. reference. The buffer used is the Tris-borate buffer described therein. DNA fragments are visualized by staining with 0.5 µg/ml ethidium bromide, which is either present in the gel and tank buffer during electrophoresis or added following electrophoresis. DNA is visualized by illumination with short-wavelength or long-wavelength ultraviolet light. When the fragments are to be isolated from the gel, the agarose used is the low gelling-temperature variety, obtained from Sigma Chemical, St. Louis, Missouri. After electrophoresis, the desired fragment is excised, placed in a plastic tube, heated to 65°C for approximately 15 minutes, then extracted with phenol three times and precipitated with ethanol twice. This procedure is slightly modified from that described in the Maniatis et al. reference at page 170.

D. Addition of synthetic linker fragments to DNA ends

When it is desired to add a new restriction endonuclease site to the end of a DNA molecule, that molecule is first treated with DNA polymerase to create flush ends, if necessary, as described in the section above.

Approximately 0.1 to 1.0 µg of this fragment is added to approximately 100 ng of phosphorylated linker DNA, obtained from New England Biolabs, in a volume of 20 to 30 µl containing 2 µl of T4 DNA ligase, from New England Biolabs, and 1 mM ATP in the buffer recommended by the manufacturer. After incubation overnight at 15°C, the reaction is terminated by heating the mixture at 65°C for ten minutes. The reaction mixture is then diluted to approximately 100 µl in a buffer suitable for the restriction endonuclease that cleaves at the synthetic linker sequence, and approximately 50 to 200 units of this endonuclease are added. The mixture is incubated at the appropriate temperature for 2 to 6 hours, then the fragment is subjected to agarose gel electrophoresis and the fragment purified as described above. The resulting fragment will now have ends with termini produced by digestion with the restriction endonuclease. These termini are usually cohesive, so that the resulting fragment is now easily ligated to other fragments having the same cohesive termini.

E. Removal of 5'-terminal phosphates from DNA fragments

During plasmid cloning steps, treatment of the vector plasmid with phosphatase reduces recircularization of the vector (discussed on page 13 of Maniatis et al. reference). After digestion of the DNA with the appropriate restriction endonuclease, one unit of calf intestine alkaline phosphatase, obtained from Boehringer-Mannheim, Indianapolis, IN, is added. The DNA is incubated at 37°C for one hour, then extracted twice with phenol and precipitated with ethanol.

F. Ligation of DNA fragments

When fragments having complementary cohesive termini are to be joined, approximately 100 ng of each fragment are incubated in a reaction mixture of 20 to 40 µl containing approximately 0.2 units of T4 DNA ligase from New England Biolabs in the buffer recommended by the manufacturer. The incubation is conducted for 1 to 20 hours at 15°C. When DNA fragments having flush ends are to be joined, they are incubated as above, except the amount of T4 DNA ligase is increased to 2 to 4 units.

G. Transformation of DNA into *E. coli*

E. coli strain HB101 is used for most experiments. DNA is introduced into *E. coli* using the calcium chloride procedure described by Maniatis et al. on pages 250-251. Transformed bacteria are selectively able to grow on medium containing appropriate antibiotics. This selective ability allows the desired bacteria to be distinguished from host bacteria not receiving transforming DNA. Determining what antibiotic is appropriate is routine, given knowledge of the drug resistance genes present on incoming transforming DNA and the drug sensitivity of the host bacteria. For example, where the host bacterium is known to be sensitive to ampicillin and there is a functional drug resistance gene for ampicillin on the incoming transforming DNA, ampicillin is an appropriate antibiotic for selection of transformants.

H. Screening *E. coli* for plasmids

Following transformation, the resulting colonies of *E. coli* are screened for the presence of the desired plasmid by a quick plasmid isolation procedure. Two conventient procedures are described on pages 366-369 of Maniatis et al. reference.

I. Large scale isolation of plasmid DNA

Procedures for isolating large amounts of plasmids in *E. coli* are found on pages 88-94 of the Maniatis et al. reference.

J. Cloning into M13 phage vectors.

In the following description, it is understood that the double-stranded replicative form of the phage M13 derivatives is used for routine procedures such as restriction endenuclease digestions, ligations, etc.

Example 1: Construction of chimeric gene in plasmid pBR322

In order to fuse the CaMV gene VI promoter and protoxin coding sequences, a derivative of phage vector mp19 (Yanish-Perron et al., 1985) is constructed.

First, a DNA fragment containing approximately 155 nucleotides 5' to the protoxin coding region and the adjacent approximately 1346 nucleotides of the coding sequence are inserted into mp19. Phage mp19 ds rf (double-stranded replicative form) DNA is digested with restriction endonucleases SacI and SmaI and the approximately 7.2 kbp (kilo base pairs) vector fragment is purified after electrophoresis through low-temperature gelling agarose by standard procedures. Plasmid pKU25/4, containing approximately 10 kbp of *Bt* DNA, including the protoxin gene, is obtained from Dr. J. Nüesch, CIBA-GEIGY Ltd., Basle, Switzerland. The nucleotide sequence of the protoxin gene present in plasmid pKU25/4 is shown in formula I. Plasmid pKU25/4 DNA is digested with endonucleases HpaI and SacI, and a 1503 bp fragment (containing nucleotides 2 to 1505 in formula (I)) is purified as above. (This fragment contains approximately 155 bp of bacterial promoter sequences and approximately 1346 bp of the start of the protoxin coding sequence). Approximately 100 ng of each fragment is then mixed, T4 DNA ligase added, and incubated at 15°C overnight. The resulting mixture is transformed into *E. coli* strain HB101, mixed with indicator bacteria *E. coli* JM 101 and plated as described (Messing, 1983). One phage called mp19/bt is used for further construction below (Figure 1).

Next, a fragment of DNA containing the CaMV gene VI promoter, and some of the coding sequences for gene VI, is inserted into mp19/bt. Phage mp19/bt ds rf DNA is digested with BamHI, treated with the large fragment of DNA polymerase to create flush ends and recleaved with endonuclease PstI. The larger vector fragment is purified by electrophoresis as described above. Plasmid pABD1 is described in Paszkowski et al., 1984. Plasmid pABD1 DNA is digested with PstI and HindIII. The fragment approximately 465 bp long containing the CaMV gene VI promoter and approximately 75 bp of the gene VI coding sequence is purified. The two fragments are ligated and plated as described above. One of the resulting recombinant phages, called mp19/btca is used in the following experiment.

Phage mp19/btca contains CaMV gene VI promoter sequences, a portion of the gene VI coding sequence, approximately 155 bp of *Bt* DNA upstream of the protoxin coding sequence, and approximately 1346 bp of the protoxin

coding sequence. To fuse the CaMV promoter sequences precisely to the protoxin coding sequences, the intervening DNA is deleted using oligonucleotide-directed mutagenesis of mp19/btca DNA. A DNA oligonucleotide with the sequence (5') TTCGGATTGTTATCCATGGTTGGAGGTCTGA (3') is synthesized by routine procedures using an Applied Biosystems DNA Synthesizer. This oligonucleotide is complementary to those sequences in phage mp19/btca DNA at the 3' end of the CaMV promoter (nucleotides 5762 to 5778 in Hohn et al., 1982) and the beginning of the protoxin coding sequence (nucleotides 156 to 172 in formula I above). The general procedure for the mutagenesis is that described in Zoller and Smith (1983). Approximately 5 µg of single-stranded phage mp19/btca DNA is mixed with 0.3 µg phosphorylated oligonucleotide in a volume of 40 µl. The mixture is heated to 65°C for 5 minutes, cooled to 50°C, and slowly cooled to 4°C. Next, buffer, nucleotide triphosphates, ATP, T4 DNA ligase and the large fragment of DNA polymerase are added and incubated overnight at 15°C as described (Zoller and Smith, 1983). After agarose gel electrophoresis, circular double-stranded DNA is purified and transfected into *E. coli* strain JM101. The resulting plaques are screened for sequences that hybridize with ^{32}P-labelled oligonucleotide, and phage are analyzed by DNA restriction endonuclease analysis. Among the resulting phage clones will be ones which have correctly deleted the unwanted sequences between the CaMV gene VI promoter and the protoxin coding sequence. This phage is called mp19/btca/del (see Figure 2).

Next, a plasmid is constructed in which the 3' coding region of the protoxin gene is fused to CaMV transcription termination signals. First, plasmid pABD1 DNA is digested with endonucleases BamHI and BglII and a 0.5 kbp fragment containing the CaMV transcription terminator sequences is isolated. Next plasmid pUC19 (Yanish-Perron et al., 1985) is digested with BamHI, mixed with the 0.5 kbp fragment and incubated with T4 DNA ligase. After transformation of the DNA into *E. coli* strain HB101, one of the resulting clones, called plasmid p702, is obtained, which has the structure shown in Figure 3.

Next, plasmid p702 DNA is cleaved with endonucleases SacI and SmaI, and the larger, approximately 3.2 kbp fragment is isolated by gel electrophoresis. Plasmid pKU25/4 DNA is digested with endonucleases AhaIII and

SacI, and the 2.3 kbp fragment (nucleotides 1502 to 3773 in formula I above) containing the 3' portion of the protoxin coding sequence (nucleotides 1504 to 3773 of the sequence shown in formula I) is isolated after gel electrophoresis. These two DNA fragments are mixed, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid is p702/bt (see Figure 3).

Finally, portions of phage mp19/btca/del ds rf DNA and plasmid p702/bt are joined to create a plasmid containing the complete protoxin coding sequence flanked by CaMV promoter and terminator sequences. Phage mp19/bt ca/del DNA is digested with endonucleases SacI and SphI, and a fragment of approximately 1.75 kbp is purified following agarose gel electrophoresis. Similarly, plasmid p702/bt DNA is digested with endonucleases SacI and SalI and a fragment of approximately 2.5 kbp is isolated. Finally, plasmid pBR322 DNA (Bolivar et al., 1977) is digested with SalI and SphI and the larger 4.2 kbp fragment isolated. All three DNA fragments are mixed and incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid, pBR322/bt14 is a derivative of pBR322 containing the CaMV gene VI promoter and translation start signals fused to the *Bt* crystal protein coding sequence, followed by CaMV transcription termination signals (shown in Figure 4).

Example 2: Construction of a Ti plasmid-derived vector.

The vector pCIB10 (Rothstein et al., 1987) is a Ti-plasmid-derived vector useful for transfer of the chimeric gene to plants via *Agrobacterium tumefaciens*. The vector is derived from the broad host range plasmid pRK252, which may be obtained from Dr. W. Barnes, Washington University, St. Louis, Mo. The vector also contains a gene for kanamycin resistance in *Agrobacterium*, from Tn903, (Oka et al., 1981) and left and right T-DNA border sequences from the Ti plasmid pTiT37. Between the border sequence are the polylinker region from the plasmid pUC18 and a chimeric gene that confers kanamycin resistance in plants.

First, plasmid pRK252 is modified to replace the gene conferring tetracycline-resistance with one conferring resistance to kanamycin from the transposon Tn903, and is also modified by replacing the unique EcoRI site in pRK252 with a BglII site (see Figure 5 for a summary of these modifi-

cations). Plasmid pRK252 is first digested with endonucleases SalI and SmaI, then treated with the large fragment of DNA polymerase I to create flush ends, and the large vector fragment purified by agarose gel electrophoresis. Next, plasmid p368, which contains Tn903 on an approximately 1050 bp BamHI fragment, is digested with endonuclease BamHI, treated with the large fragment of DNA polymerase, and an approximately 1050 bp fragment is isolated after agarose gel electrophoresis; this fragment contains the gene from transposon Tn903 which confers resistance to the antibiotic kanamycin (Oka et al., 1981). Both fragments are then treated with the large fragment of DNA polymerase to create flush ends. Both fragments are mixed and incubated with T4 DNA ligase overnight at 15°C. After transformation into *E. coli* strain HB101 and selection for kanamycin resistant colonies, plasmid pRK252/Tn903 is obtained (see Figure 5).

Plasmid pRK252/Tn903 is digested at its unique EcoRI site, followed by treatment with the large fragment of *E. coli* DNA polymerase to create flush ends. This fragment is added to synthetic BglII restriction site linkers, and incubated overnight with T4 DNA ligase. The resulting DNA is digested with an excess of BglII restriction endonuclease and the larger vector fragment purified by agarose gel electrophoresis. The resulting fragment is again incubated with T4 DNA ligase to recircularize the fragment via its newly added BglII cohesive ends. Following transformation into *E. coli* strain HB101, plasmid pRK252/Tn903/BglII is obtained (see Figure 5).

A derivative of plasmid pBR322 is constructed which contains the Ti plasmid T-DNA borders, the polylinker region of plasmid pUC19, and the selectable gene for kanamycin resistance in plants (see Figure 6). Plasmid pBR325/Eco29 contains the 1.5 kbp EcoRI fragment from the nopaline Ti plasmid pTiT37. This fragment contains the T-DNA left border sequence (Yadav et al., 1982). To replace the EcoRI ends of this fragment with HindIII ends, plasmid pBR325/Eco29 DNA is digested with EcoRI, then incubated with nuclease S1, followed by incubation with the large fragment of DNA polymerase to create flush ends, then mixed with synthetic HindIII linkers and incubated with T4 DNA ligase. The resulting DNA is digested with endonucleases ClaI and an excess of HindIII, and the

resulting 1.1 kbp fragment containing the T-DNA left border is purified by gel electrophoresis. Next, the polylinker region of plasmid pUC19 is isolated by digestion of the plasmid DNA with endonucleases EcoRI and HindIII and the smaller fragment (approx. 53 bp) is isolated by agarose gel electrophoresis. Next, plasmid pBR322 is digested with endonucleases EcoRI and ClaI, mixed with the other two isolated fragments, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid, pCIB5, contains the polylinker and T-DNA left border in a derivative of plasmid pBR322 (see Figure 6).

A plasmid containing the gene for expression of kanamycin resistance in plants is constructed (see Figures 7 and 8). Plasmid pBIN6 is obtained from Dr. M. Bevan, Plant Breeding Institute, Cambridge, UK. This plasmid is described in the reference by Bevan, 1984. Plasmid pBIN6 DNA is digested with EcoRI and HindIII and the fragment approximately 1.5 kbp in size containing the chimeric neomycin phosphotransferase (NPT) gene is isolated and purified following agarose gel electrophoresis. This fragment is then mixed with plasmid pUC18 DNA which has been cleaved with endonucleases EcoRI and HindIII. Following incubation with T4 DNA ligase, the resulting DNA is transformed into *E. coli* strain HB101. The resulting plasmid is called pUC18/neo. This plasmid DNA contains an unwanted BamHI recognition sequence between the neomycin phosphotransferase gene and the terminator sequence of nopaline synthase gene (see Bevan, 1984). To remove this recognition sequence, plasmid pUC18/neo is digested with endonuclease BamHI, followed by treatment with the large fragment of DNA polymerase to create flush ends. The fragment is then inubated with T4 DNA ligase to recircularize the fragment, and is transformed into *E. coli* strain HB101. The resulting plasmid, pUC18/neo (Bam) has lost the BamHI recognition sequence.

The T-DNA right border sequence is then added next to the chimeric NPT gene (see Figure 8). Plasmid pBR325/Hind23 contains the 3.4 kbp HindIII fragment of plasmid pTiT37. This fragment contains the right T-DNA border sequence (Bevan et al., 1983). Plasmid pBR325/Hind23 DNA is cleaved with endonucleases SacII and HindIII, and a 1.0 kbp fragment containing the right border is isolated and purified following agarose gel electrophoresis. Plasmid pUC18/neo(Bam) DNA is digested with endonucleases SacII

and HindIII and the 4.0 kbp vector fragment is isolated by agarose gel electrophoresis. The two fragments are mixed, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid, pCIB4 (shown in Figure 8), contains the T-DNA right border and the plant-selectable marker for kanamycin resistance in a derivative of plasmid pUC18.

Next, a plasmid is constructed which contains both the T-DNA left and right borders, with the plant selectable kanamycin-resistance gene and the polylinker of pUC18 between the borders (shown in Figure 9). Plasmid pCIB4 DNA is digested with endonuclease HindIII, followed by treatment with the large fragment of DNA polymerase to create flush ends, followed by digestion with endonuclease EcoRI. The 2.6 kbp fragment containing the chimeric kanamycin-resistance gene and the right border of T-DNA is isolated by agarose gel electrophoresis.

Plasmid pCIB5 DNA is digested with endonuclese AatII, treated with T4 DNA polymerase to create flush ends, then cleaved with endonuclease EcoRI. The larger vector fragment is purified by agarose gel electrophoresis, mixed with the pCIB4 fragment, incubated with T4 DNA ligase, and transformed into *E. coli* strain HB101.

The resulting plasmid, pCIB2 (shown in Figure 9) is a derivative of plasmid pBR322 containing the desired sequences between the two T-DNA borders.

The following steps complete the construction of the vector pCIB10, and are shown in Figure 10. Plasmid pCIB2 DNA is digested with endonuclease EcoRV, and synthetic linkers containinig BglII recognition sites are added as described above. After digestion with an excess of BglII endonuclease, the approximately 2.6 kbp fragment is isolated after agarose gel electrophoresis. Plasmid pRK252/Tn903/BglII, described above (see Figure 5), is digested with endonuclease BglII and then treated with phosphatase to prevent recircularization. These two DNA fragments are mixed, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid is the completed vector, pCIB10.

Example 3: Insertion of the chimeric protoxin gene into vector pCIB10.
The following steps are shown in Figure 11. Plasmid pBR322/bt14 DNA is digested with endonucleases PvuI and SalI, and then partially digested with endonuclease BamHI. A BamHI-SalI fragment approx. 4.2 kbp in size, containing the chimeric gene, is isolated following agarose gel electrophoresis, and mixed with plasmid pCIB10 DNA which has been digested with endonucleases BamHI and SalI. After incubation with T4 DNA ligase and transformation into *E. coli* strain HB101, plasmid pCIB10/19Sbt is obtained (see Figure 11). This plasmid contains the chimeric protoxin gene in the plasmid vector pCIB10.

In order to transfer plasmid pCIB10/19Sbt from *E. coli* HB101 to *Agrobacterium*, an intermediate *E. coli* host strain S17-1 (Simon et al., 1983) is used. This strain, obtainable from Agrigenetics Research Corp., Boulder, Co., contains mobilization functions that can transfer plasmid pCIB10/19Sbt directly to *Agrobacterium* via conjugation, thus avoiding the necessity to transform naked plasmid DNA directly into *Agrobacterium*. First, plasmid pCIB10/19Sbt DNA is introduced into calcium chloride-treated S17-1 cells. Next, cultures of transformed S17-1 cells and *Agrobacterium tumefaciens* strain LBA 4404 (Ooms et al.,1982) are mixed and mated on an N agar (Difco) plate overnight at room temperture. A loopful of the resulting bacteria are streaked onto AB minimal media, (Chilton et al., 1974) plated with 50 µg/ml kanamycin and incubated at 28°C. Colonies are restreaked onto the same media, then restreaked onto N agar plates. Slow-growing colonies are picked, restreaked onto AB minimal media with kanamycin and single colonies isolated. This procedure selects for Agrobacteria containing the pCIB10/19Sbt plasmid.

Example 4: Transfer of the chimeric gene to tobacco plant cells.
Protoplasts of *Nicotiana tabacum* cv. "Coker 176" are prepared as follows: Four to five week old shoot cultures are grown aseptically in MS medium (Murashige and Skoog, 1962) without hormones at 26°C with a 16 hour light/8 hour dark photoperiod. Approximately 1.5 g leaf tissue are removed from the plant and distributed equally among 8 to 10 Petri dishes (100 X 25 mm, Lab-Tek), each containing 10 ml of enzyme solution. Enzyme solution contains 1 % cellulase R-10 (Yakult Pharmaceutical Co.), 0.25 % macerase (Calbiochem Co.), 1 % pectolyase Y-23 (Seishin Pharma-

ceutical Co.), 0.45 M mannitol and 0.1 x K3 salts (Nagy and Maliga, 1976). Tobacco leaves are cut into thin strips with a scalpel, the dishes are sealed, placed on a gyrotory shaker at 35 rpm and incubated with the enzymes for 4 to 5 hours at room temperature.

Next, contents of the dishes are filtered through a cheesecloth-lined funnel and collected in a flask. The filtrate is pipetted into babcook flasks containing 35 ml each of rinse solution. [Rinse solution contains 0.45 M sucrose, MES (2-[N-morpholino]ethanesulfonic acid), and 0.1 x K3 salts.] The bottles are centrifuged at 80 x g for ten minutes, after which the protoplasts will have floated to the top of the bottle. The protoplasts are removed with a 1 ml pipet, combined into one bottle, and rinsed twice more. The resulting protoplasts are suspended in K3 medium in a 15 ml disposable centrifuge tube.

Concentration of protoplasts is determined by counting in a Fuchs-Rosenthal hemocytometer. Protoplasts are then plated at a density of 100,000/ml in 6 ml of liquid K3 medium per 100 x 20 mm Petri dish (Corning). The dishes containing the protoplasts are incubated at 26°C in the dark for two days, during which time cell wall regeneration will occur.

After the two-day incubation, 5 µl of a stationary culture of *A. tumefaciens* containing pCIB10/19Sbt are added to the dish of protoplasts. (The Agrobacteria are grown in YEP medium plus 50 µg/ml kanamycin at 28°C until stationary phase is reached.) After incubation for three more days at 26°C, cefotaxime (Calbiochem Co.) is added (500 µg/ml) to kill the Agrobacteria. The following day, cells are diluted with 3 ml fresh K3 medium per dish, and cefotaxime added again (500 µg/ml). Cells are then grown at 26°C for 2 to 3 weeks and then screened on selective medium as described by DeBlock et al. (1984).

Example 5: Construction of a *Bt* protoxin chimeric gene with the CaMV 35S promoter.

5.1. Construction of a CaMV 35S Promoter Cassette

Plasmid pCIB710 is constructed as shown in Figure 12. This plasmid contains CaMV promoter and transcription termination sequences for the

35S RNA transcript (Covey et al., 1981). A 1149 bp BglII restriction fragment of CaMV DNA [bp 6494-7643 in Hohn et al., 1982] is isolated from plasmid pLV111 (obtained from Dr. S. Howell Univ. California-San Diego). Alternatively, the fragment can be isolated directly from CaMV DNA by preparative agarose gel electrophoresis as described earlier and mixed with BamHI-cleaved plasmid pUC19 DNA, treated with T4 DNA ligase, and transformed into *E. coli*. (Note the BamHI restriction site in the resulting plasmid has been destroyed by ligation of the BglII cohesive ends to the BamHI cohesive ends.) The resulting plasmid, called pUC19/35S, is then used in oligonucleotide- directed in-vitro mutagenesis to insert the BamHI recognition sequence GGATCC immediately following CaMV nucleotide 7483 in the Hohn reference. The resulting plasmid, pCIB710, contains the CaMV 35S promoter region and transcription termination region separated by a BamHI restriction site. DNA sequences inserted into this BamHI site will be expressed in plants by these CaMV transcription regulation sequences. (Also note that pCIB710 does not contain any ATG translation initiation codons between the start of transcription and the BamHI site.)

5.2. Insertion of the CaMV 35S promoter/Terminator Cassette into pCIB10.

The following steps are outlined in Figure 13. Plasmids pCIB10 and pCIB710 DNAs are digested with EcoRI and SalI, mixed and ligated. The resulting plasmid, pCIB10/710 has the CaMV 35S promoter/terminator cassette inserted into the plant transformation vector pCIB10. The CaMV 35S sequences are between the T-DNA borders in pCIB10, and thus will be inserted into the plant genome in plant transformation experiments.

5.3. Insertion of the *Bt* protoxin gene into pCIB10/710

The following steps are outlined in Figure 14. As a source of the protoxin gene, plasmid pCIB10/19Sbt is digested with BamHI and NcoI, and the 3.6 kbp fragment containing the protoxin gene is isolated by preparative gel electrophoresis. The fragment is then mixed with synthetic NcoI-BamHI adapter with the sequence 5'-CATGGCCGGATCCGGC-3', then digested with BamHI. This step creates BamHI cohesive ends at both ends of the protoxin fragment. This fragment is then inserted into BamHI-

cleaved pCIB10/710. The resulting plasmid, pCIB10/35Sbt, shown in Figure 14, contains the protoxin gene between the CaMV 35S promoter and transcription termination sequences.

5.4. Transfer of the plasmid pCIB10/35Sbt into *Agrobacterium tumefaciens* for plant transformation.

The plasmid pCIB10/35Sbt is transferred into *A. tumefaciens* strain LBA4404 as described in example 4, above.

Example 6: Construction of pTOX, containing a chimeric gene encoding the insecticidal toxin gene of *Bt* var. *tenebrionis*

A gene encoding the insecticidal crystal protein gene of *Bt* var. *tenebrionis* has been characterized and sequenced (Sekar et al., 1987). This coding sequence is isolated on a convenient restriction fragment, such as a HindIII fragment of approximately 3 kbp in size, and inserted into an appropriate plant expression vector, such as the plasmid pCIB770 (Rothstein et al., 1987). The plasmid pCIB770 contains a chimeric kanamycin gene for expression in plants, as well as the promoter and terminator of the 35S RNA transcript of CaMV separated by a unique BamHI site. The restriction fragment bearing the toxin coding sequence is made compatible to the unique BamHI site of pCIB770 by use of the appropriate molecular adapter and ligated together.

Example 7: Construction of pSAN, containing a chimeric gene encoding the insecticidal toxin gene of *Bt* strain *san diego*

A gene encoding the insecticidal protein of *Bt* strain *san diego* has been characterized and sequenced by Herrnstadt et al., EP-0-202-739 and EP-0-213-818. This coding sequence is isolated on a convenient restriction fragment and inserted into the appropriate plant expression vector, such as pCIB770. The plasmid pCIB770 contains a chimeric kanamycin gene for expression in plants, as well as the promoter and terminator of the 35S RNA transcript of CaMV separated by a unique BamH site. The restriction fragment bearing the toxin coding sequence is made compatible to the unique BamHI site of pCIB770 by use of the appropriate molecular adapter and ligated together.

Example 8: Construction of a deleted *Bt* protoxin gene encoding a polypeptide of approximately 725 amino acids, and construction of a chimeric gene containing this deleted gene with the CaMV 35S promoter

A deleted protoxin gene encoding a polypeptide of approximately 725 amino acids is made by removing the COOH-terminal portion of the gene by cleaving at the KpnI restriction endonuclease site at position 2325 in the sequence shown in formula I.

Plasmid pCIB10/35Sbt (Figure 14) is digested with BamHI and KpnI, and the approximately 2.2 kbp BamHI/KpnI fragment containing the deleted protoxin gene is isolated by preparative agarose gel electrophoresis. To convert the KpnI site at the 3' end to a BamHI site, the fragment is mixed with a KpnI/BamHI adapter oligonucleotide and ligated. This fragment is then mixed with BamHI-cleaved pCIB10/710 (Figure 13). The resulting transformants, designed pCIB10/35Sbt (KpnI) and shown in Figure 15, contain the deleted protoxin gene encoding a polypeptide of approximately 725 amino acids. These transformants are selected on kanamycin.

Example 9: Construction of a deleted *Bt* protoxin gene encoding a polypeptide of approximately 645 amino acids, and construction of a chimeric gene containing this deleted gene with the CaMV 35S promoter.

A deleted protoxin gene encoding a polypeptide of approximately 645 amino acids is made by removing the COOH-terminal portion of the gene by cleaving at the BclI restriction endonuclease site at position 2090 in the sequence shown in Formula I.

Plasmid pCIB10/35Sbt (Figure 14) is digested with BamHI and BclI, and the approximately 1.9 kbp BamHI/BclI fragment containing the deleted protoxin gene is isolated by preparative agarose gel electrophoresis. Since BclI creates a cohesive end compatible with BamHI, no further manipulation is required prior to ligating this fragment into BamHI-cleaved pCIB10/710 (Figure 13). The resulting plasmid pCIB10/35Sbt(BclI) which has the structure shown in Figure 16, is selected on kanamycin.

Example 10: Construction of a deleted *Bt* protoxin gene encoding a polypeptide of approximately 607 amino acids, and construction of a chimeric gene containing this deleted gene with the CaMV 35S promoter.

A deleted protoxin gene is made by introducing a BamHI cleavage site (GGATCC) following nucleotide 1976 in the sequence shown in Formula I.

This is done by cloning the BamHI fragment containing the protoxin sequence from pCIB10/35Sbt into mp18, and using standard oligonucleotide mutagenesis procedures described above. After mutagenesis, double-stranded replicative form DNA is prepared from the M13 clone, which is then digested with BamHI. The approximately 1.9 kbp fragment containing the deleted protoxin gene is inserted into BamHI-cleaved pCIB10/710. The resulting plasmid pCIB10/35Sbt(607) which has the structure shown in Figure 17, is selected for on kanamycin.

The remaining Examples describe specific protocols for transforming cotton cells and regenerating cotton plants from cotton cells and callus. It should be understood that those with ordinary skill in the art may vary the details of the protocols while still remaining within the limits of the present invention. For example, numerous plant tissue culture media are known, some of which are described in detail below. The ordinarily skilled tissue culture scientist would know how to vary these solutions in order to achieve the same or similar results. Thus, Example 12 discloses a modified White's stock solution as a seed germination and callus development medium; Example 13 describes a Murashige and Skoog stock solution as a callus growth/maintenance medium; Example 14 describes a Beasley and Ting stock solution as a plant germination medium. The ordinarily skilled tissue culture scientist knows how to vary these solutions in order to achieve results similar to those described in the Examples. Thus, the sugar in the callus growth medium may be glucose, which minimizes phenolic secretions, or sucrose, which promotes the formation of embryogenic callus.

The explants used in the transformation procedure may be from any suitable source, such as from seedlings, especially a hypocotyl or cotyledon, or from immature embryos of developing fruit.

Any antibiotic toxic to *Agrobacterium* may be used to kill residual *Agrobacterium* after the transformation step. Cefotaxime is preferred.

Example 11: Regeneration of cotton plants

11.1. Media

All media in this example contain Murashige and Skoog inorganic salts and Gamborg's B-5 vitamins, are adjusted to pH 5.7, and have the following composition (mg/liter):

Macronutrients	
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
KNO_3	1900
NH_4NO_3	1650
$CaCl_2 \cdot 2H_2O$	440

Micronutrients	
H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	15.6
$ZnSO_4 \cdot 7H_2O$	8.6
$NaMoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CaCl_2 \cdot 6H_2O$	0.025
KI	0.83
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3

Vitamines	
Thiamine • HCl	10
Pyridoxine • HCl	1
Nicotinic acid	1
Myo-Inositol	100

In addition, the various media have the following components:

Medium #	Additional Components
1	20 g/liter sucrose, 0.6 % noble agar (Difco)
2	30 g/liter glucose, 2 mg/liter α-naphthaleneacetic acid 1 mg/liter kinetin, 0.8 % noble agar
3	30 g/liter sucrose, 2 mg/liter α-naphthaleneacetic acid 1 mg/liter kinetin, 0.8 % noble agar
4	20 g/liter sucrose, 0.5 mg/liter picloram
5	20 g/liter sucrose, 5 mg/liter 2,4-dichlorphenoxyacetic acid
6	20 g/liter sucrose, 15 mM glutamine

Media at 25°, 28° and 31°C refer, in addition to the temperature, to a photoperiod of 16 hours light: 8 hours dark at a light intensity of 20 $\mu E\ m^{-2}s^{-1}$ (= 1670 lx).

11.2. Seed Sterilization and Planting

Seeds of cotton (*Gossypium hirsutum* var. Coker 310) are delinted by placing seed in concentrated H_2SO_4 for 2 min. Seeds are then washed 4 times with sterile, distilled water, dipped in 95 % ethanol, flamed and planted on Medium #1 at 31°C.

11.3. Callus induction

Seven days following planting, seedling hypocotyls are excised, sliced longitudinally, cut into 2 mm sections and placed on Medium #2 at 31°C. Hypocotyl sections (2 mm) are transferred weekly to fresh Medium #2 and these cultures are also maintained at 31°C. Following 4 weekly transfers to Medium #2, callus tissue proliferating on the hypocotyl sections is removed from the original explant and placed on Medium #3 at 31°C. The callus is transferred to fresh Medium #3 after one month and maintained for an additional 1 to 2 months.

11.4. Suspension Culture Initiation

For initiation of suspension cultures, 100 mg of callus tissue is placed into 35 ml of Medium #4 in a 125 ml DeLong flask. Suspensions are rotated for 6 weeks at 140 rpm, and 28°C, at which time they begin rapidly to proliferate.

11.5. Embryo Development and Plant Regeneration

The embryos that form in Medium #4 proliferate even faster following replacement of Medium #4 by Medium #5. This embryogenic suspension is divided and subcultured every 3 to 7 days into fresh Medium #5. For development of embryos proliferating in Medium #5, the embryos are washed with, and then placed into, Medium #6. Three to four weeks following transfer to Medium #6, the mature embryos are placed on a solid medium at 25°C. The solid medium consists of a modified MS medium containing MS salts with 40 mM KNO_3 in place of KNO_3 and NH_4NO_3, B-5 vitamins, 2 % sucrose, 15 mM glutamine, and solidified with 0.2 % Gelrite (pH 5.7). Embryos are placed in petri dishes at 25°C. Shoot development is sporadic on this medium and root elongation is enhanced with the transfer of the embryos to the above modified MS medium without glutamine. Germinating embryos are then planted in vermiculite in pots and covered with a beaker (25°C). After plantlets are established in vermiculite, the beaker is removed. Following one week at 28°C, the plantlets are placed in the greenhouse for further development into plants.

Example 12: Seed germination and callus development media

[Composition of modified White (1961)'s stock solution (incorporated herein by reference)]

Component	Concentration per 1000 ml.	Comments
$MgSO_4 \cdot 7\ H_2O$	3.6 g	Dissolve and make up the final volume to 1000 ml. Label White's A Stock. Use 100 ml/liter of final medium
Na_2SO_4	2.0 g	
$NaH_2PO_4 \cdot H_2O$	1.65 g	
$Ca(NO_3)_2 \cdot 4\ H_2O$	2.6 g	Dissolve and make up the final volume to 1000 ml. Label White's B Stock. Use 100 ml/liter of final medium.
KNO_3	800 mg	
KCl	650 mg	
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg	Dissolve and make up the final volume to 100 ml. Label White's C Stock. Use 1.0 ml/liter final medium.
$CoCl_2 \cdot 6H_2O$	2.5 mg	
$MnSO_4 \cdot H_2O$	300 mg	
$ZnSO_4 \cdot 7\ H_2O$	50 mg	
$CuSO_4 \cdot 5\ H_2O$	2.5 mg	
H_3BO_3	50 mg	
Fe-EDTA		Use 10 ml/liter of MSFe EDTA. (See below)
Organic		Use 10 ml/liter of MS organic. (See below)

Example 13: Callus growth/maintenance media

[Composition of Murashige & Skoog (MS) (1962, stock solutions (incorporated herein by reference)]

Component	Concentration per 1000 ml. of stock		Comments
NH_4NO_3	41.26	g	Dissolve and make up the final volume to 1000 ml. Label MS-Major. Use 40 ml/liter of final medium
KNO_3	47.50	g	
$CaCl_2 \cdot 2\ H_2O$	11.00	g	
$MgSO_4 \cdot 7\ H_2O$	9.25	g	
KH_2PO_4	4.25	g	
KI	83	mg	Dissolve and make up the final volume to 1000 ml. Label MS-Minor. Use 100 ml/liter of final medium.
H_3BO_3	620	mg	
$MnSO_4 \cdot H_2O$	1690	mg	
$ZnSO_4 \cdot 7\ H_2O$	860	mg	
$Na_2MoO_4 \cdot 2\ H_2O$	25	mg	
$CuSO_4 \cdot 5\ H_2O$	2.5	mg	
$CoCl_2 \cdot 6\ H_2O$	2.5	mg	
Nicotinic acid	50	mg	Dissolve and make up the final volume to 1000 ml. Label MS-Organic. Freeze in 10 ml aliquots. Use 10 ml/liter of final medium.
Pyridoxine HCl	50	mg	
Thiamine HCl	10	mg	

Component	Concentration per 1000 ml. of stock	Comments
Fe EDTA Fe $SO_4 \cdot 7H_2O$ Na_2 EDTA$\cdot$2 H_2O	2.78 g 3.73 g	Dissolve 2.78 g of $FeSO_4 \cdot 7\ H_2O$ in about 200 ml of deionized water. Dissolve 3.73 g of Na_2-EDTA$\cdot$2 H_2O (disodium salt of ethylenediaminotetraacetic acid dihydrate) in 200 ml of deionized water in another beaker. Heat the Na_2-EDTA solution on a hot plate for about 10 minutes. While constantly stirring, add $FeSO_4$ solution to Na_2-EDTA solution. Cool the solution to room temperature and make up the volume to 1000 ml. Label MSFe-EDTA. Cover bottle with foil and store in refrigerator. Use 10 ml/liter of final medium.
Thiamine HCl	50 mg	Dissolve and make up the volume to 500 ml. Label MS - Thiamine. Use 4.0 ml/liter of final medium.
m-Inositol Glycine	10 g 0.2 g	Dissolve and make up the final volume to 1000 ml. Label MS - glycine/inositol. Use 10 ml/liter of final medium.

Example 14: Plant germination media

[Composition of Beasley and Ting (1973)'s stock solutions]

Component	Concentration per 1000 ml.	Comments
KH_2PO_4	2.72 g	Dissolve and make up the volume to 100 ml. Label B & T - A Stock. Use 10 ml/l of final medium.
H_3BO_3	61.83 mg	
$Na_2MoO_4 \cdot 2\ H_2O$	2.42 mg	
$CaCl_2 \cdot 2\ H_2O$	2.6 g	Dissolve and make up the volume to 100 ml. Label B & T - B Stock. Use 10 ml/l of final medium.
KI	8.3 mg	
$CoCl_2 \cdot 6\ H_2O$	0.24 mg	
$MgSO_4 \cdot 7\ H_2O$	4.93 g	Dissolve and make up the volume to 100 ml. Label B & T - C Stock. Use 10 ml/l of final medium.
$MnSO_4 \cdot H_2O$	169.02 mg	
$ZnSO_4 \cdot 7\ H_2O$	86.27 mg	
$CuSO_4 \cdot 5\ H_2O$	0.25 mg	
KNO_3	25.275 g	Dissolve and make up the volume to 200 ml. Label B & T - D Stock. Use 40 ml/l of final medium.
Nicotinic acid	4.92 mg	Dissolve and make up the final volume to 100 ml. Label B & T - Organics. Use 10 ml/l of final medium.
Pyridoxine HCl	8.22 mg	
Thiamine HCl	13.49 mg	
Fe-EDTA		Use 10 ml/l of MS-Fe-EDTA.
Inositol		100 mg/l of final medium.
NH_4NO_3 (15 µM)		1200.6 mg/l of final medium.

Example 15: Regeneration of plants starting from cotyledon explants

Seeds of Acala cotton variety SJ2 of *Gossypium hirsutum* are sterilized by contact with 95 % alcohol for three minutes, then twice rinsed with sterile water and immersed with a 15 % solution of sodium hypochlorite for 15 minutes, then rinsed in sterile water. Sterilized seeds are germinated on a basal agar medium in the dark for approximately 14 days to produce a seedling. The cotyledons of the seedlings are cut into segments of 2 to 4 mm^2 which are transferred aseptically to a callus inducing medium [see above] consisting of Murashige and Skoog (MS) major and minor salts supplemented with 0.4 mg/liter thiamine-HCl, 30 g/liter glucose, 2.0 mg/liter naphthaleneacetic acid (NAA), 1 mg/liter kinetin, 100 mg/liter m-inositol, and agar (0.8 %). The cultures are incubated at about 30°C under conditions of 16 hours light and 8 hours darkness in a Percival incubator with fluorescent lights (cool daylight) providing a light intensity of about 2000 to 4000 lx.

Calli are formed on the cultured tissue segments within 3 to 4 weeks and are white to gray-greenish in color. The calli formed are subcultured every three to four weeks onto a callus growth medium comprising MS medium containing 100 mg/liter m-inositol, 20 g/liter sucrose, 2 mg/liter naphthaleneacetic acid (NAA) and agar. Somatic embryos form four to six months after first placing the tissue explants on the callus inducing medium. The callus and embryos are maintained on callus growth medium by subculturing onto fresh callus growth medium every three to four weeks.

Somatic embryos which formed on tissue pieces are explanted either to fresh callus growth medium, or to Beasley & Ting's medium (embryo germination medium).

The somatic plantlets which are formed from somatic embryos are transferred onto Beasley and Ting's medium which contains 1200 mg/liter ammonium nitrate and 500 mg/liter casein hydrolysate as an organic nitrogen source. the medium is solidified by a solidifying agent (Gelrite) and plantlets are placed in Magenta boxes.

The somatic embryos develop into plantlets within about three months. The plantlets are rooted at the six to eight leaf stage [about 7.5 and 10 cm tall], and transferred to soil and maintained in an incubator under high humidity for three to four weeks, after which they are transferred to the greenhouse. After hardening, plants are transferred to open tilled soil.

Example 16: Regeneration of plants starting from cotyledon explants - Variation 1

The procedure of Example 15 is repeated using instead half-strength MS medium in which all medium components have been reduced to one-half the specified concentration. Essentially the same results are obtained.

Example 17: Regeneration of different cotton varieties from cotyledon explants.

The procedure of Examples 15 and 16 is repeated with Acala cotton varieties SJ4, SJ2C-1, GC510, B1644, B2724, B1810, the picker variety Siokra and the stripper variety FC2017. All are successfully regenerated.

Example 18: Regeneration of cotton plants from cotyledon explants with suspension cell culture as intermediate step.

The procedure of Example 15 is repeated to the extent of obtaining callus capable of forming somatic embryos.

Pieces of about 750 to 1000 mg of actively growing embryogenic callus are suspended in 8 ml units of liquid suspension culture medium comprised of MS major and minor salts, supplemented with 0.4 mg/liter thiamine HCl, 20 g/liter sucrose, 100 mg/liter m-inositol and naphthaleneacetic acid (2 mg/liter) in T-tubes and placed on a roller drum rotating at 1.5 rpm under 16:8 light:dark regime. Light intensity of about 2000 to 4500 lx is again provided by fluorescent lights (cool daylight).

After four weeks, the suspension is filtered through an 840 micron size nylon mesh to remove larger cell clumps. The fraction smaller than 840 microns is allowed to settle, washed once with about 20 to 25 ml of fresh suspension culture medium. This cell suspension is transferred to T-tubes (2 ml per tube) and each tube diluted with 6 ml of fresh suspension culture medium. The cultures are maintained by repeating the

above at 10 to 12 day intervals. At each subculture, the suspension is filtered and only the fraction containing cell aggregates smaller than 840 microns is transferred to fresh suspension culture medium. In all instance, the fraction containing cell clumps larger than 840 microns are placed onto the callus growth medium to obtain mature somatic embryos.

The somatic embryos that are formed on callus growth medium are removed and transferred to embryo germination medium. Using the protocol of Example 15, these are germinated, developed into plantlets and then field grown plants.

Example 19: Regeneration of cotton plants from cotyledon explants with suspension cell culture as an intermediate step-Variant 1.

The procedure of Example 18 is repeated except that suspension cultures are formed by transferring 750 to 1000 mg embryogenic calli to a DeLong flask containing 15 to 20 ml of the MS liquid medium containing 2 mg/liter NAA. The culture containing flask is placed on gyrotory shaker and shaken at 100 to 110 strokes/minute. After three weeks the suspension is filtered through an 840 micron nylon mesh to remove the large cell clumps for plant growth, as in Example 18. The less than 840 micron suspension is allowed to settle, washed once in the MS liquid medium and resuspended in 2 to 5 ml of the MS liquid medium. The suspension is subcultured by transfer to fresh medium in a DeLong flask containing 1 to 2 ml of suspension and 15 ml of fresh MS liquid medium. The cultures are maintained by repeating this procedure at seven to ten day intervals. At each subculture only the less than 840 micron suspensions are subcultured and the large clumps (840 microns or greater) used for plant growth.

Example 20: Production of plants from large clumps of suspension cultured cells

After three or four subcultures using the suspension growth procedure of Examples 18 and 19, 1.5 ml to 2.0 ml of cell suspension from the T-tube and DeLong flask are in each instance plated onto agar-solidified MS medium containing 2 mg/liter NAA and Beasley & Ting medium containing 500 mg/liter casein hydrolysate. Within three to four weeks embryogenic calli with developing embryos become visible. Again, the 840 micron or

greater cell clumps are plated on the callus growth medium, give rise to embryogenic clumps with developing embryos, which ultimately grow into plants.

Example 21: Transformation of cotton suspension culture cells to tumorous-phenotype by Agrobacteria LBA 4434.

21.1. Growth of the plant suspension culture.

An Acala cotton suspension culture [as described in Example 18, above] is subcultured into "T" tubes with the medium (MS medium containing 2 mg/liter NAA) being changed every seven to ten days. After a medium change, the "T" tube is rotated 90° and the cells allowed to settle out. The supernatant is removed by pipeting prior to transformation and the resulting cells treated as described below.

21.2. Description of *Agrobacterium* vector.

The *Agrobacterium* strain LBA 4434 (Hoekema et al., 1983) contains a Ti plasmid-derived binary plant transformation system. In such binary systems, one plasmid contains the T-DNA of a Ti-plasmid, the second plasmid contains the vir-region of a Ti-plasmid, and together the two plasmids function to effect plant transformation. In the *Agrobacterium* strain LBA 4434, the T-DNA plasmid pAL1050 contains T_L of pTiAch5, an octopine Ti-plasmid. The vir plasmid in strain LBA 4434, pAL4404, contains the intact virulence regions of pTiAch5 (Ooms et al., 1982). Strain LBA 4434 is available from Dr. Robert Schilperoort of the Department of Biochemistry, University of Leiden, the Netherlands.

21.3. Growth of Agrobacteria.

The transforming *Agrobacterium* strain is taken from a glycerol stock, inoculated in a small overnight culture, from which a 50 ml culture is inoculated the following day. Agrobacteria are grown on YEB medium [YEB is per liter in water: 5 g beef extract, 1 g yeast extract, 5 g peptone, 5 g sucrose, adjusted to pH 7.2 with NaOH. After autoclaving, 1 ml of 2 M $MgCl_2$ is added] to which antibiotics as appropriate have been added. The absorbance at 600 nm (OD_{600}) of the 50 ml overnight culture is read, the culture is centrifuged and the pellet resuspended in the plant cell

growth medium (MS medium plus NAA at 2 mg/ml) to a final absorbance at 600 nm of 0.5. 8 ml of this bacterial suspension is added to each "T" tube containing the plant cells from part 21.1 above.

21.4. Infection.

The "T"tube containing the plant and bacteria cells is agitated to resuspend all cells and returned to a roller drum for three hours to allow the Agrobacteria to attach to the plant cells. The cells are then allowed to settle and the residual supernatant removed. A fresh aliquot of growth medium is added to the "T" tube and this allowed to incubate on a roller drum for a period of 18 to 20 hours in the presence of any residual Agrobacteria which remained. After this time, the cells are again allowed to settle, the supernatant is removed and the cells are washed twice with a solution of growth medium containing cefotaxime (200 µg/ml). After washing, the cells from each T-tube are resuspended in 10 ml growth medium containing cefotaxime (200 µg/ml in all cases) and 1 ml aliquots of this plated on petri dishes.

21.5. Growth of transformed tissue.

The cells infected with Agrobacteria grow on the growth medium which had no added phytohormones, indicating the tissue has received the wild-type phytohormone genes in T-DNA. These cells develop into tumors, further indicating transformation of the cultures.

Example 22: Transformation of cotton suspension culture cells to a kanamycin-resistant non-tumorous phenotype.

The same procedure as in Example 21 is followed except that different transforming Agrobacteria are used and that the plant selection medium contains an antibiotic for the selection of transformed plant tissue.

22.1. Growth of plant tissue.

As in Example 21, part 21.1.

22.2. Description of *Agrobacterium* vector.

The transforming Agrobacteria contain the T-DNA containing binary vector pCIB10 (Rothstein et al., 1987) as well as the pAL4404 vir plasmid.
The T-DNA of pCIB10 contains a chimeric gene composed of the promoter

from nopaline synthase, the coding region from Tn5 [encoding the enzyme neomycin phosphotransferase], and the terminator from nopaline synthase. The *Agrobacterium* strain LBA4404, containing the vir helper plasmid pAL4404 [described above], is similarly available from Dr. Schilperoort.

22.3. Growth of Agrobacteria.

Agrobacteria containing pCIB10 are grown on YEB containing kanamycin (50 µg/ml). Otherwise, conditions are as in Example 21, part 21.3.

22.4. Infection.

Transformation is accomplished as detailed in Example 21 with the change that the 1 ml aliquots resulting in part 21.3 are plated immediately on medium containing selective antibiotics. Selection medium contained either kanamycin (50 µg/ml) or G418 (25 µg/ml). Expression of the nos/neo/nos chimeric gene in transformed plant tissue allows the selection of this tissue on either of these antibiotics.

22.5. Growth of transformed tissue.

Plant growth media in this and all following examples contain phytohormones as indicated in Example 15.

In 2 to 4 weeks, transformed tissue becomes apparent on the selection plates. Uninfected tissue or control tissue shows no signs of growth, turns brown and dies. Transformed tissue grows very well in the presence of kanamycin or G418. At this time, tissue pieces which are growing well are subcultured to fresh selection medium.

22.6. Growth of Somatic Embryos.

Somatic embryos form on these tissue pieces. Somatic embryos are explanted to fresh medium (non selective).

22.7. Germination.

When the embryos have begun to differentiate and germinate, i.e. the point where they are beginning to form roots and had two or three leaves, they are transferred to Magenta boxes containing growth medium. Growth is allowed to proceed until the plantlet has 6 to 8 leaves, at which time it is removed from the agar medium.

22.8. Growth of plantlet.

The plantlet is now placed in potting soil, covered with a beaker to maintain humidity and placed in a Percival incubator for 4 to 8 weeks. At this time, the beaker is removed and the plant transferred to the greenhouse.

22.9. Growth of plant in greenhouse.

The plants grow in the greenhouse, flower and set seed.

Example 23: Transformation of cotton suspension culture cells to a glyphosate-tolerant phenotype

The same procedure as in Example 22 is followed except where changes are noted below. Different transforming Agrobacteria are used. Also, after plant tissue is selected on an antibiotic for the selection of transformed material, it is further selected for herbicide tolerance.

23.1. Growth of plant tissue

As in Example 21, part 21.1.

23.2. Description of *Agrobacerium* vector.

Transforming Agrobacteria contain the T-DNA vector pPMG85/587 (Fillatti et al., 1987) as well as the pAL4404 vir plasmid. The plasmid pPMG85/587 carries three chimeric genes capable of expression in plants. Two of these genes code for neomycin phsphotransferase (NPT) which confers resistance to antibiotic kanamycin or G418. The third chimeric gene, containing the coding sequence from a mutant aroA gene of *Salmonella typhimurium*, confers tolerance to the herbicide glyphosate (Comai et al., 1983).

23.3. Growth of Agrobacteria.

Agrobacteria containing pPMG85/587 are grown on medium containing kanamycin (100 µg/ml).

23.4. Infection.

Transformation is accomplished as detailed in Example 21 with the change that the 1 ml aliquots resulting in part 21.3 are plated immediately on medium containing selective antibiotics. This selceton medium contains either kanamycin (50 µg/ml) or G418 (25 µg/ml). Expression of the NPT chimeric gene in transformed plant tissue allows the selection of this tissue on either of these antibiotics.

23.5. Growth of transformed tissue.

In 2 to 4 weeks, transformed tissue becomes apparent on the selection plates. Plant material is originally selected on kanamycin.

Plant tissue [either individual embryos or callus] is then placed on medium containing the herbicide glyphosate. Transformed tissue continues to grow well.

Example 24: Transformation of cotton suspension culture cells to a hygromycin-resistant non-tumorous phenotype.

The same procedure as in Example 22 is followed except where noted. Different transforming Agrobacteria are used and the plant selection medium contains an antibiotic appropriate for the selection of transformed plant tissue.

24.1. Growth of plant tissue.

As in Example 21, part 21.1.

24.2. Description of *Agrobacterium*.

The transforming Agrobacteria contain the T-DNA containing binary vector pCIB2115 (Rothstein et al., 1987) as well as the vir plasmid. The T-DNA of pCIB2115 contains a chimeric gene composed of the promoter and terminator from the CaMV 35S transcript [Odell et al., 1985] and the coding sequence for hygromycin B phosphotransferase [Gritz and Davies, 1983].

24.3. Growth of Agrobacteria.

Agrobacteria containing pCIB2115 are grown on YEB containing kanamycin (50 µg/ml).

24.4. Infection.

Transformation is accomplished as detailed in Example 21 with the change that the 1 ml aliquots resulting in part 21.3 are plated immediately on medium containing selective antibiotics. Selection medium contains 50 µg/ml hygromycin. Expression of the chimeric hygromycin gene in transformed plant tissue allows the selection of this tissue on medium containing hygromycin.

24.5. Growth of transformed tissue.

As in Example 22, part 22.5 except that the antibiotic hygromycin is used in the plant selection growth medium.

Example 25: Plant extraction procedure

Plant tissue is homogenized in extraction buffer [ca 100 mg in 0.1 ml Extraction Buffer].

Leaf extraction buffer

Na_2CO_3 (pH 9.5)	50 mM
EDTA	10 mM
Triton X-100	0.05 %
Tween	0.05 %
NaCl	1000mM
PMSF (add just prior to use)	1 mM
leupeptine (add just prior to use).	1 mM

After extraction, 2 M Tris pH 7.0 is added to adjust the pH of the extract to a pH of 8.0 to 8.5. The extract is then centrifuged 10 minutes in a Beckman microfuge and the supernatant used for ELISA analysis.

Example 26: ELISA analysis of plant tissue

ELISAs [enzyme-linked immunosorbent assay] are very sensitive, specific assays for antigenic material. ELISAs are very useful for studying the expression of polypeptide gene products. The development of ELISA techniques as a general tool is described by Clark et al. (1986); this is herein incorporated by reference.

An ELISA for the *Bt* toxin was developed using standard procedures and is used to analyze transgenic plant material for expression of *Bt* sequences. The steps used in this procedure are as given below:

Media and Buffers

EPBS (ELISA Phosphate Buffered Saline)

10 mM Na Phosphate:	Na_2HPO_4	4.68 g/4 liter.
	$NaH_2PO_4 \cdot H_2O$	0.976 g/4 liter
140 mM NaCl	NaCl	32.7 g/4 liter

pH should be approximately 7.4

Borate Buffered Saline

100 mM Boric acid

25 mM Na Borate

75 mM NaCl

Adjust pH to 8.4 to 8.5 with HCl or NaOH as needed.

ELISA Blocking Buffer

In EPBS,

1 % BSA

0.02 % Na Azide

ELISA Wash Buffer

10 mM Tris-HCl pH 8.0

0.05 % Tween 20

0,02 % Na Azide

2.5 M TRIS

FLISA Diluent

In EPBS:

0.05 % Tween 20

1 % BSA

0.02 % Na Azide

ELISA Substrate Buffer

In 500 ml,

48 ml Diethanolamine,

24.5 mg $MgCl_2$;

adjust to pH 9.8 with HCl.

ELISA Substrate

15 mg p-nitrophenyl phosphate in 25 ml substrate buffer.

Procedure:

1. ELISA plate is pretreated with ethanol.
2. Affinity-purified rabbit anti-*Bt* toxin antiserum (50 µl) at a concentration of 3 µg/ml in Borate Buffered Saline is added to the plate and this allowed to incubate overnight at 4°C. Antiserum is produced in response to immunizing rabbits with gradient-purified *Bt* toxin crystals (Ang and Nickerson, 1978) solubilized with sodium dodecyl sulfate.
3. Wash with ELISA Wash Buffer.
4. Treat 1 hour at room temperature with Blocking Buffer.
5. Wash with ELISA Wash Buffer.
6. Add plant extract in an amount to give 50 µg of protein (this is typically ca. 5 µl of extract). Leaf extraction buffer is described in example 25; protein is determined by the Bradford method (Bradford, 1976) using a commercially available kit [Bio-Rad, Richmond, California]. If dilution of the leaf extract is necessary, ELISA Diluent is used. Allow this to incubate overnight at 4°C.
7. Wash with ELISA Wash Buffer.

8. Add 50 µl affinity-purified goat anti-*Bt* toxin antiserum at a concentration of 3 µg protein/ml in ELISA Diluent. Allow this to incubate for one hour at 37°C.
9. Wash with ELISA Wash Buffer.
10. Add 50 µl rabbit anti-goat antibody bound to alkaline phosphatase [commercially available from Sigma Chemicals, St. Lous, Mo.]. This is diluted 1:500 in Diluent. Allow to incubate for one hour at 37°C.
11. Wash with ELISA Wash Buffer.
12. Add 50 µl substrate [0.6 mg/ml p-nitrophenyl phosphate in ELISA Substrate Buffer. Incubate for 30 minutes at room temperature.
13. Terminate reaction by adding 50 µl of 3 M NaOH.
14. Read absorbance at 405 nm in modified ELISA reader [Hewlett Packard, Stanford, Ca.].

Plant tissue transformed with the pCIB10/35Sbt(BclI) [see Figure 16] construction, when assayed using this ELISA procedure shows a positive reaction, indicating expression of the *Bt* gene.

Example 27: Bioassay of transformed cotton

Heliothis virescens eggs laid on sheets of cheesecloth are obtained from the Tobacco Insect Control Laboratory at North Carolina State University, Raleigh, North Carolina. The cheesecloth sheets are transferred to a large covered glass beaker and incubated at 29°C with wet paper towels to maintain humidity. The eggs hatch within three days. As soon as possible after hatching, the larvae (one larva per cup) are transferred to covered small plastic cups. Each cup contains cotton leaf discs. Larvae are transferred using a fine bristle paint brush.

Leaf discs one cm in diameter are punched from leaves of cotton plants and placed on a circle of wet filter paper in the cup with the larva. At least 6 to 10 leaf discs, representing both young and old leaves, are tested from each plant. Leaf discs are replaced at two day intervals, or as necessary to feed the larvae. Growth rates [size or combined weight of all replica worms] and mortality of larvae feeding on leaves of transformed plants are compared with those of larvae feeding on untransformed cotton leaves.

Larvae feeding on discs of cotton transformed with pCIB 10/35Sbt (BclI) show a decrease in growth rate and an increase in mortality compared with controls.

Example 28: Construction of pCIB1300, for high level expression in plants.

pCIB1300 is engineered for high level expression of the *Bt* toxin gene and contains an untranslated leader sequence 5' to the *Bt* toxin gene to enhance *Bt* toxin gene expression in plants. The untranslated leader is a 40 bp sequence 5' to the initiation codon of the *Bt* toxin gene and 3' to the CaMV 35S untranslated leader. The final pCIB1300 construct is engineered by the insertion of the 40 bp leader and deleted *Bt* toxin gene into the BamH1 site of pCIB10/710 as shown in Figure 19. A 1.9 kbp NcoI-BamHI fragment from pCIB10/35Sbt(Bcl) deletion is purified in low-temperature gelling agarose. The 40 bp leader is chemically synthesized as a double-stranded oligonucleotide with a 5' overhanging BamHI site and a 3' overhanging NcoI site using an Applied Biosystems DNA Synthesizer. The sequence of the untranslated leader as shown in the center of Figure 19 is derived from the alfalfa mosaic virus (AMV) coat protein untranslated leader described by Koper-Zwarthoff et al. (1977). The 40 bp leader, 1.9 kbp *Bt* fragment and BamHI linearized pCIB710 vector are joined in a three-part ligation using T4 DNA ligase to construct pCIB1300.

Example 29: Isolation of cDNA clones coding for the small subunit of RuBPCase in Cotton

Gossypium hirsutum (Funk line RF522) plants are grown from seeds in the greenhouse with 14 hour daily light periods. Total RNA is isolated from young green leaves following the procedure of Newbury and Possingham (1977). PolyA$^+$ RNA is purified as described in Maniatis et al. (1982), p. 197. Double-stranded cDNA (complementary DNA) is synthesized according to the procedure of Okayama and Berg (1982) with the following modifications:

A. First strand cDNA is primed with oligo-dT;
B. After tailing the double-stranded cDNA with oligo-dG using poly-nucleotidyl-transferase, it is cloned into oligo-dC tailed pUC9 (Pst I site - from Pharmacia), and annealed; and
C. the DNA is transformed into *E. coli* strain HB101.

Since, with the chlorophyll a/b binding protein (Cab), RuBPCase is the most abundant protein in green leaves, the cDNA library is then screened for cDNA clones of the most abundant mRNAs. Nitrocellulose (Schleicher and Schuell) filter replicas of the cDNA clones are screened with the first cDNA strand, radioactively labeled with α-dCT^{32}P and reverse-transcriptase, the template being the same polyA$^+$ RNA as that used to construct the cDNA library. Six cDNA clones out of 275, are selected and analyzed further.

Northern analysis (done as described in Maniatis et al., 1982, p. 202) shows that two of these cDNA clones hybridize to a class of mRNA about 1100 nt long. They cross-hybridize with a Cab gene probe from tobacco. The other four hybridize to a class of mRNA 900 to 1000 nt long, a size consistent with that of the rbcS (small subunit of Rubisco). Cotton leaf mRNA, after hybrid selection using one of these four cDNA clones, is released and translated in vitro (as described in Maniatis et al. 1982, p. 329) using rabbit reticulocytes in vitro translation kit (Promega Biotec). Electrophoresis on polyacrylamide gel of the translation products showed one major polypeptide of about 20 kD, a molecular weight consistent with that of the precursor of the RuBPCase The other 3 cDNA clones cross-hybridize with the clone used for the hybrid-release experiment.

Large portions of these cDNA clones are sequenced, using the dideoxy chain-termination technique (Sanger et al., 1977) after subcloning into M13. Comparison of their sequences with formerly published rbcS sequences from other species shows that they are indeed rbcS cDNA clones.

Example 30: Isolation of genomic clones of small subunit RuBPcase of cotton

30.1. Cotton Genomic Southern analysis.

Genomic Southern blots are prepared by standard procedures using nitrocellulose filters. Prehybridization, hybridization and washing conditions are as described in Klessig et al. (1983). Genomic Southern analysis, using our rbcS cDNA clone as a probe, reveals 4 to 5 genomic fragments depending on the restriction enzyme used to digest the DNA. The RuBPCase is encoded by a small gene family in cotton, as in other species previously studied by others. The cotton rbcS multigene family is estimated to contain at least 5 members.

30.2. Isolation of rbcS genomic clones

In order to construct a cotton genomic library, partial Sau3a digests of cotton genomic DNA are size-fractionated on a 10 % to 40 % sucrose-gradient, and ligated into λ EMBL3 arms (Stratagene) digested with BamHI. Packaging of λ recombinants, done using Packagene kit (Stratagene), is followed by transfection into *E. coli* strain K802. Nitrocellulose filter duplicate replicas are screened as described in Maniatis et al. (1982) p. 320, using the rbcS cDNA clone from above as a probe. Twelve positive clones out of 450,000 plaques are purified. DNA is isolated form plate lysates of these recombinants phages, as described in Maniatis et al. (1982) p. 80.

After comparing these genomic clones by their restriction digest pattern with various enzymes, five different rbcS genes are identified. Each one is subcloned into the plasmid vector pBSM13^{+} (Stratagene). These subclones are then mapped and partially sequenced in order to localize the 5' end of the gene and the first ATG (translational start site). A map of two of these genomic subclones, rbc-gX and rbc-gY is shown on Figure 24. The λ EMBL3 phages containing the genomic DNA of subclones rbc-gX and rbc-gY have been deposited with the International Depository American Type Culture Collection, Rockville, Maryland.

30.3. Study of the level of expression of the rbcS gene fragments in cotton leaves

Forty-one additional rbcS cDNA clones are isolated from the cotton leaf cDNA library. Restriction mapping analysis, sequencing and hybridization of these cDNA clones to gene specific probes allows to conclude that the gene carried by the genomic clone rbc-gX is responsible for about 17 % of the cotton leaf rbcS transcripts.

Example 31: Construction of chimeric genes using cotton rbcS promoter.

31.1. Insertion of an Nco I site at the first ATG of the rbcS genes encoding transit peptides.

The sequences of the transit peptides of rbc-gX and rbc-gY are shown on Figures 26 and 25 respectively. An Nco I cleavage site (CCATGG) is introduced at the first ATG of these two genes of the encoding transit peptide. This is done by cloning the PstI-EcoRI fragment of gene rbc-gX and the XbaI-SphI fragment of gene rbc-gY (hatched fragments on Figures 22 and 23 respectively) into mp18 and mp19 respectively, and using standard oligonucleotide site-directed mutagenesis procedures described above to introduce the NcoI site.

31.2. Construction of pCIB 1301, a plasmid bearing a chimeric gene containing the deleted *Bt* protoxin gene (607 deletion) with the rbc-gX gene promoter.

After the site-directed mutagenesis, double-stranded replicative form (ds rf) DNA is isolated from the M13 clone, which is then digested with Hind III and Eco RI. The Hind III-Eco RI fragment containing the rbc-gX promoter is ligated together with Hind III and Eco RI digested plasmid pUC19 and the ligation mix then transformed into *E. coli* strain HB101. Plasmid DNA is isolated from ampicillin-selected transformants and digested with HindIII. The ends of the resulting molecule are made blunt-ended by treatment with the Klenow subunit of DNA polymerase I and Sal I linkers are ligated to these ends. The resulting linear molecule is digested with Sal I and Nco I and gel-purified. In a three-part ligation the gel-purified Sal I-Nco I fragment is joined to a gel-purified Bam HI-Sal I fragment from pCIB770, a broad-host range replicon used as

an *Agrobacterium* Ti plasmid cloning vector (Rothstein et al., 1987) and a gel-purified Nco I-Bam HI fragment containing the truncated 607 amino acid Bt gene. The ligation mix is transformed into *E. coli* strain HB101. The resulting plasmid, pCIB1301, which is depicted graphically in Figures 20, 21 and 22, is selected on kanamycin.

31.3. Construction of pCIB1302, a plasmid bearing a chimeric gene containing the deleted *Bt* protoxin gene (607 deletion) with the rbc-gY gene promoter.

After the mutagenesis, double-stranded replicative form (ds rf) DNA is isolated from the M13 clone, which is then digested with Xba I-Nco I. The approximately 1.97 kbp NcoI-BamHI fragment, containing the deleted protoxin gene, is then ligated, together with the XbaI-NcoI rbc-gY promoter fragment, in a three way ligation, into XbaI-BamHI cleaved pCIB10/710. The resulting plasmid, pCIB1302, the structure of which is shown in Figure 23, is selected on kanamycin.

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The claims defining the invention are as follows:

1. A cotton cell comprising a chimeric gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein, exhibiting toxicity toward Dipteran and Lepidopteran insects.

2. The cell according to claim 1 wherein the plant cells are cells of *Gossypium hirsutum, Gossypium arboreum, or Gossypium barbadense*.

3. The cell according to claim 2 wherein the plant cells are cells of *Gossypium hirsutum*.

4. The cell according to claim 1 wherein the plant cells are of the variety Acala SJ-2, Acala GC 510, Acala B-1644 or Siokra.

5. The cell according to any one of claims 1 to 4 wherein the promoter, 5' untranslated region, and, optionally the 3' untranslated region of the chimeric gene are derived from plant or plant virus genes.

6. The cell according to claim 5 wherein the promoter, 5' untranslated region and/or optionally 3' untranslated region of the chimeric gene are derived from a plant gene that codes for the small subunit of ribulose-1,5-bisphosphate carboxylase or chlorophyll a/b-binding protein.

7. The cell according to claim 5 or 6 wherein the promoter, 5' untranslated region and/or optionally 3' untranslated region are derived from a gene of a plant DNA virus.

8. The cell of claim 7 wherein the plant virus is cauliflower mosaic virus.

9. The cell of claim 8 wherein the cauliflower mosaic virus promoter is the 35S promoter of gene VI.

10. The cell of claim 1 wherein the promoter, 5' untranslated region and optionally the 3' untranslated region of the chimeric gene are derived from DNA sequences that are present in *Agrobacterium* plasmids, and that cause expression in plants.

11. The cell of claim 10 wherein the promoter is derived from the Ti plasmid of *Agrobacterium tumefaciens*.

12. The cell of claim 10 wherein said DNA sequences are derived from a gene that codes for octopine synthase.

13. The cell of claim 10 wherein said DNA sequences are derived from a gene that codes for nopaline synthase.

14. The cell of claim 1 wherein the polypeptide has an Mr of about 130,000 to about 140,000, or insecticidal fragments thereof.

15. The cell of claim 14 wherein the polypeptide is fused to another molecule.

16. The cell of claim 1 wherein the chimeric gene is substantially complementary to the nucleotide sequence that codes for the crystal protein δ-endotoxin in *Bacillus thuringiensis*.

17. The cell of claim 1 wherein the chimeric gene is capable of hybridizing to the coding region of the gene that codes for the crystal protein δ-endotoxin in *Bacillus thuringiensis*.

18. The cell of claim 14 wherein the polypeptide has substantially the same immunological properties as the crystal protein from *Bacillus thuringiensis*.

19. The cell of claim 16, 17 or 18 wherein said *Bacillus thuringiensis* is a subspecies selected from the group consisting of *Bt* var. *kurstaki*, *Bt* var. *berliner*, *Bt* var. *alesti*, *Bt* var. *tolworthi*, *Bt* var. *sotto*, *Bt* var. *dendrolimus*, *Bt* var. *tenebrionis*, *Bt* var. *san diego* and *Bt* var. *aizawai*.

20. The cell of claim 19 wherein the *Bacillus thuringiensis* is the variety *kurstaki* HD1.

21. The cell of claim 20 wherein the gene expresses a polypeptide having the amino acid sequence:

Sequence of the formula (II)

Met Asp Asn Asn Pro Asn Ile Asn Glu Cys	10
Ile Pro Tyr Asn Cys Leu Ser Asn Pro Glu	20
Val Glu Val Leu Gly Gly Glu Arg Ile Glu	30
Thr Gly Tyr Thr Pro Ile Asp Ile Ser Leu	40
Ser Leu Thr Gln Phe Leu Leu Ser Glu Phe	50
Val Pro Gly Ala Gly Phe Val Leu Gly Leu	60
Val Asp Ile Ile Trp Gly Ile Phe Gly Pro	70
Ser Gln Trp Asp Ala Phe Leu Val Gln Ile	80
Glu Gln Leu Ile Asn Gln Arg Ile Glu Glu	90
Phe Ala Arg Asn Gln Ala Ile Ser Arg Leu	100
Glu Gly Leu Ser Asn Leu Tyr Gln Ile Tyr	110
Ala Glu Ser Phe Arg Glu Trp Glu Ala Asp	120
Pro Thr Asn Pro Ala Leu Arg Glu Glu Met	130
Arg Ile Gln Phe Asn Asp Met Asn Ser Ala	140
Leu Thr Thr Ala Ile Pro Leu Phe Ala Val	150
Gln Asn Tyr Gln Val Pro Leu Leu Ser Val	160
Tyr Val Gln Ala Ala Asn Leu His Leu Ser	170
Val Leu Arg Asp Val Ser Val Phe Gly Gln	180
Arg Trp Gly Phe Asp Ala Ala Thr Ile Asn	190
Ser Arg Tyr Asn Asp Leu Thr Arg Leu Ile	200
Gly Asn Tyr Thr Asp His Ala Val Arg Trp	210
Tyr Asn Thr Gly Leu Glu Arg Val Trp Gly	220
Pro Asp Ser Arg Asp Trp Ile Arg Tyr Asn	230
Gln Phe Arg Arg Glu Leu Thr Leu Thr Val	240
Leu Asp Ile Val Ser Leu Phe Pro Asn Tyr	250
Asp Ser Arg Thr Tyr Pro Ile Arg Thr Val	260
Ser Gln Leu Thr Arg Glu Ile Tyr Thr Asn	270
Pro Val Leu Glu Asn Phe Asp Gly Ser Phe	280
Arg Gly Ser Ala Gln Gly Ile Glu Gly Ser	290
Ile Arg Ser Pro His Leu Met Asp Ile Leu	300
Asn Ser Ile Thr Ile Tyr Thr Asp Ala His	310
Arg Gly Glu Tyr Tyr Trp Ser Gly His Gln	320
Ile Met Ala Ser Pro Val Gly Phe Ser Gly	330
Pro Glu Phe Thr Phe Pro Leu Tyr Gly Thr	340
Met Gly Asn Ala Ala Pro Gln Gln Arg Ile	350
Val Ala Gln Leu Gly Gln Gly Val Tyr Arg	360
Thr Leu Ser Ser Thr Leu Tyr Arg Arg Pro	370
Phe Asn Ile Gly Ile Asn Asn Gln Gln Leu	380
Ser Val Leu Asp Gly Thr Glu Phe Ala Tyr	390
Gly Thr Ser Ser Asn Leu Pro Ser Ala Val	400
Tyr Arg Lys Ser Gly Thr Val Asp Ser Leu	410
Asp Glu Ile Pro Pro Gln Asn Asn Asn Val	420
Pro Pro Arg Gln Gly Phe Ser His Arg Leu	430
Ser His Val Ser Met Phe Arg Ser Gly Phe	440
Ser Asn Ser Ser Val Ser Ile Ile Arg Ala	450

Pro Met Phe Ser Trp Ile His Arg Ser Ala	460
Glu Phe Asn Asn Ile Ile Pro Ser Ser Gln	470
Ile Thr Gln Ile Pro Leu Thr Lys Ser Thr	480
Asn Leu Gly Ser Gly Thr Ser Val Val Lys	490
Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu	500
Arg Arg Thr Ser Pro Gly Gln Ile Ser Thr	510
Leu Arg Val Asn Ile Thr Ala Pro Leu Ser	520
Gln Arg Tyr Arg Val Arg Ile Arg Tyr Ala	530
Ser Thr Thr Asn Leu Gln Phe His Thr Ser	540
Ile Asp Gly Arg Pro Ile Asn Gln Gly Asn	550
Phe Ser Ala Thr Met Ser Ser Gly Ser Asn	560
Leu Gln Ser Gly Ser Phe Arg Thr Val Gly	570
Phe Thr Thr Pro Phe Asn Phe Ser Asn Gly	580
Ser Ser Val Phe Thr Leu Ser Ala His Val	590
Phe Asn Ser Gly Asn Glu Val Tyr Ile Asp	600
Arg Ile Glu Phe Val Pro Ala Glu Val Thr	610
Phe Glu Ala Glu Tyr Asp Leu Glu Arg Ala	620
Gln Lys Ala Val Asn Glu Leu Phe Thr Ser	630
Ser Asn Gln Ile Gly Leu Lys Thr Asp Val	640
Thr Asp Tyr His Ile Asp Gln Val Ser Asn	650
Leu Val Glu Cys Leu Ser Asp Glu Phe Cys	660
Leu Asp Glu Lys Lys Glu Leu Ser Glu Lys	670
Val Lys His Ala Lys Arg Leu Ser Asp Glu	680
Arg Asn Leu Leu Gln Asp Pro Asn Phe Arg	690
Gly Ile Asn Arg Gln Leu Asp Arg Gly Trp	700
Arg Gly Ser Thr Asp Ile Thr Ile Gln Gly	710
Gly Asp Asp Val Phe Lys Glu Asn Tyr Val	720
Thr Leu Leu Gly Thr Phe Asp Glu Cys Tyr	730
Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu	740
Ser Lys Leu Lys Ala Tyr Thr Arg Tyr Gln	750
Leu Arg Gly Tyr Ile Glu Asp Ser Gln Asp	760
Leu Glu Ile Tyr Leu Ile Arg Tyr Asn Ala	770
Lys His Glu Thr Val Asn Val Pro Gly Thr	780
Gly Ser Leu Trp Pro Leu Ser Ala Pro Ser	790
Pro Ile Gly Lys Cys Ala His His Ser His	800
His Phe Ser Leu Asp Ile Asp Val Gly Cys	810
Thr Asp Leu Asn Glu Asp Leu Gly Val Trp	820
Val Ile Phe Lys Ile Lys Thr Gln Asp Gly	830
His Ala Arg Leu Gly Asn Leu Glu Phe Leu	840
Glu Glu Lys Pro Leu Val Gly Glu Ala Leu	850
Ala Arg Val Lys Arg Ala Glu Lys Lys Trp	860
Arg Asp Lys Arg Glu Lys Leu Glu Trp Glu	870
Thr Asn Ile Val Tyr Lys Glu Ala Lys Glu	880
Ser Val Asp Ala Leu Phe Val Asn Ser Gln	890
Tyr Asp Arg Leu Gln Ala Asp Thr Asn Ile	900
Ala Met Ile His Ala Ala Asp Lys Arg Val	910
His Ser Ile Arg Glu Ala Tyr Leu Pro Glu	920
Leu Ser Val Ile Pro Gly Val Asn Ala Ala	930
Ile Phe Glu Glu Leu Glu Gly Arg Ile Phe	940
Thr Ala Phe Ser Leu Tyr Asp Ala Arg Asn	950
Val Ile Lys Asn Gly Asp Phe Asn Asn Gly	960
Leu Ser Cys Trp Asn Val Lys Gly His Val	970
Asp Val Glu Glu Gln Asn Asn His Arg Ser	980
Val Leu Val Val Pro Glu Trp Glu Ala Glu	990
Val Ser Gln Glu Val Arg Val Cys Pro Gly	1000
Arg Gly Tyr Ile Leu Arg Val Thr Ala Tyr	1010

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Lys Glu Gly Tyr Gly Glu Gly Cys Val Thr          1020
Ile His Glu Ile Glu Asn Asn Thr Asp Glu          1030
Leu Lys Phe Ser Asn Cys Val Glu Glu Glu          1040
Val Tyr Pro Asn Asn Thr Val Thr Cys Asn          1050
Asp Tyr Thr Ala Thr Gln Glu Glu Tyr Glu          1060
Gly Thr Tyr Thr Ser Arg Asn Arg Gly Tyr          1070
Asp Gly Ala Tyr Glu Ser Asn Ser Ser Val          1080
Pro Ala Asp Tyr Ala Ser Ala Tyr Glu Glu          1090
Lys Ala Tyr Thr Asp Gly Arg Arg Asp Asn          1100
Pro Cys Glu Ser Asn Arg Gly Tyr Gly Asp          1110
Tyr Thr Pro Leu Pro Ala Gly Tyr Val Thr          1120
Lys Glu Leu Glu Tyr Phe Pro Glu Thr Asp          1130
Lys Val Trp Ile Glu Ile Gly Glu Thr Glu          1140
Gly Thr Phe Ile Val Asp Ser Val Glu Leu          1150
Leu Leu Met Glu Glu End                          1156
```

22. The cell of claim 1 wherein the DNA sequence of the coding region of the gene comprises:

Formula I

```
          10         20         30         40         50         60
GTTAACACCC TGGGTCAAAA ATTGATATTT AGTAAAATTA GTTGCACTTT GTGCATTTTT

          70         80         90        100        110        120
TCATAAGATG AGTCATATGT TTTAAATTGT AGTAATGAAA AACAGTATTA TATCATAATG

         130        140        150        160        170        180
AATTGGTATC TTAATAAAAG AGATGGAGGT AACTTATGGA TAACAATCCG AACATCAATG

         190        200        210        220        230        240
AATGCATTCC TTATAATTGT TTAAGTAACC CTGAAGTAGA AGTATTAGGT GGAGAAAGAA

         250        260        270        280        290        300
TAGAAACTGG TTACACCCCA ATCGATATTT CCTTGTCGCT AACGCAATTT CTTTTGAGTG

         310        320        330        340        350        360
AATTTGTTCC CGGTCCTGGA TTTGTGTTAG GACTAGTTGA TATAATATCG GGAATTTTTG

         370        380        390        400        410        420
GTCCCTCTCA ATGGGACGCA TTTCTTGTAC AAATTGAACA GTTAATTAAC CAAAGAATAG

         430        440        450        460        470        480
AAGAATTCGC TAGGAACCAA GCCATTTCTA GATTAGAAGG ACTAAGCAAT CTTTATCAAA

         490        500        510        520        530        540
TTTACGCAGA ATCTTTTAGA GAGTGGGAAG CAGATCCTAC TAATCCAGCA TTAAGAGAAG

         550        560        570        580        590        600
AGATGCGTAT TCAATTCAAT GACATGAACA GTGCCCTTAC AACCGCTATT CCTCTTTTTG
```

610 620 630 640 650 660
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670 680 690 700 710 720
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730 740 750 760 770 780
TCAATAGTCG TTATAATGAT TTAACTAGGC TTATTGGCAA CTATACAGAT CATGCTGTAC

790 800 810 820 830 840
GCTGGTACAA TACGGGATTA GAGCGTGTAT GGGGACCGGA TTCTAGAGAT TGGATAAGAT

850 860 870 880 890 900
ATAATCAATT TAGAAGAGAA TTAACACTAA CTGTATTAGA TATCGTTTCT CTATTTCCGA

910 920 930 940 950 960
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970 980 990 1000 1010 1020
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1330 1340 1350 1360 1370 1380
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1390 1400 1410 1420 1430 1440
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1450 1460 1470 1480 1490 1500
GATTAAGCCA TGTTTCAATG TTTCGTTCAG GCTTTAGTAA TAGTAGTGTA AGTATAATAA

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1570 1580 1590 1600 1610 1620
CACAAATTAC ACAAATACCT TTAACAAAAT CTACTAATCT TGGCTCTGGA ACTTCTGTCG

1630 1640 1650 1660 1670 1680
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1690 1700 1710 1720 1730 1740
CAACCTTAAG AGTAAATATT ACTGCACCAT TATCACAAAG ATATCGGGTA AGAATTCGCT

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      1810        1820        1830        1840        1850        1860
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      1870        1880        1890        1900        1910        1920
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      1930        1940        1950        1960        1970        1980
ATGTCTTCAA TTCAGGCAAT GAAGTTTATA TAGATCGAAT TGAATTTGTT CCGGCAGAAG

      1990        2000        2010        2020        2030        2040
TAACCTTTGA GGCAGAATAT GATTTAGAAA GAGCACAAAA GGCGGTGAAT GAGCTGTTTA

      2050        2060        2070        2080        2090        2100
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      2110        2120        2130        2140        2150        2160
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      2170        2180        2190        2200        2210        2220
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      2230        2240        2250        2260        2270        2280
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      2290        2300        2310        2320        2330        2340
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      2350        2360        2370        2380        2390        2400
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      2410        2420        2430        2440        2450        2460
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      2470        2480        2490        2500        2510        2520
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      2530        2540        2550        2560        2570        2580
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      2590        2600        2610        2620        2630        2640
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      2650        2660        2670        2680        2690        2700
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      2710        2720        2730        2740        2750        2760
CACTAGCTCG TGTGAAAAGA GCGGAGAAAA AATGGAGAGA CAAACGTGAA AAATTGGAAT

      2770        2780        2790        2800        2810        2820
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      2950        2960        2970        2980        2990        3000
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      3010        3020        3030        3040        3050        3060
GAAATGTCAT TAAAAATGGT GATTTTAATA ATGGCTTATC CTGCTGGAAC GTGAAAGGGC

      3070        3080        3090        3100        3110        3120
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      3130        3140        3150        3160        3170        3180
CAGAAGTGTC ACAAGAAGTT CGTGTCTGTC CGGGTCGTGG CTATATCCTT CGTGTCACAG

      3190        3200        3210        3220        3230        3240
CGTACAAGGA GGGATATGGA GAAGGTTGCG TAACCATTCA TGAGATCGAG AACAATACAG

      3250        3260        3270        3280        3290        3300
ACGAACTGAA GTTTAGCAAC TGTGTAGAAG AGGAAGTATA TCCAAACAAC ACGGTAACGT

      3310        3320        3330        3340        3350        3360
GTAATGATTA TACTGCGACT CAAGAAGAAT ATGAGGGTAC GTACACTTCT CGTAATCGAG

      3370        3380        3390        3400        3410        3420
GATATGACGG AGCCTATGAA AGCAATTCTT CTGTACCAGC TGATTATGCA TCAGCCTATG

      3430        3440        3450        3460        3470        3480
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      3490        3500        3510        3520        3530        3540
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      3550        3560        3570        3580        3590        3600
CCGATAAGGT ATGGATTGAG ATCGGAGAAA CGGAAGGAAC ATTCATCGTG GACAGCGTGG

      3610        3620        3630        3640        3650        3660
AATTACTTCT TATGGAGGAA TAATATATGC TTTATAATGT AAGGTGTGCA AATAAAGAAT

      3670        3680        3690        3700        3710        3720
GATTACTGAC TTGTATTGAC AGATAAAATAA GGAAATTTTT ATATGAATAA AAAACGGGCA

      3730        3740        3750        3760        3770        3780
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      3790        3800        3810        3820        3830        3840
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      3850        3860        3870        3880        3890        3900
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      3910        3920        3930        3940        3950        3960
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      4270        4280        4290        4300        4310        4320
TATTTCAAC GAATCCGTAT TTAGATGCG ACGATTTTCC AAGTACCGAA ACATTTAGCA

      4330        4340        4350        4360
CATGTATATC CTGGGTCAGG TGGTTGTGCA CAAACTGCAG
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23. The cell of claim 20 wherein the chimeric gene expresses an insecticidal polypeptide having substantial sequence homology to the amino acid sequence of claim 21.

24. The cell of claim 20 wherein the chimeric gene shows substantial sequence homology to the DNA sequence given in claim 22.

25. A culture of cotton cells according to any one of claims 1 to 24.

26. The culture of claim 25 wherein the cotton cells are cells of *Gossypium hirsutum, Gossypium arboreum and Gossypium barbadense*.

27. The culture according to claim 26 wherein the plant cells are cells of *Gossypium hirsutum*.

28. The culture of any one of claims 25 to 27 wherein the cells are protoplasts.

29. A cotton plant comprising a gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein, exhibiting toxicity toward Dipteran and Lepidopteran insects.

30. A cotton plant according to claim 29 comprising a gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein in sufficient amounts to render the plant unattractive and/or toxic to insect larvae.

31. A cotton plant according to claim 30 comprising a gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein in sufficient amounts to render the plant toxic to lepidopteran, dipteran and coleopteran larvae.

32. The plant of claim 29 selected from the group consisting of *Gossypium hirsutum*, *Gossypium arboreum* and *Gossypium barbadense*.

33. The plant according to claim 32 wherein the plant cells are cells of *Gossypium hirsutum*.

34. Propagules of a transgenic cotton plant according to any one of claim 29 to 33.

35. The propagules of claim 34 selected from the group consisting of protoplasts, cells, calli, tissues, embryos, organs, seeds, pollen, ovules, zygotes or any other propagules that can be obtained from a transgenic cotton plant.

36. The propagules of claim 34 that can be sexually or asexually propagated or that can be propagated in-vitro or in-vivo.

37. Progeny of a transgenic cotton plant according to any one of claims 29 to 33, or mutants and variants thereof, that still have the characteristic properties of the starting material, caused by the previous transformation of exogenous DNA.

38. A method of producing transformed, embryogenic cotton callus which comprises:

a) contacting a cotton explant with an *Agrobacterium* vector containing a chimeric gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein and a gene that confers resistance to an antibiotic on cotton cells, the period of the contacting being sufficient to transfer the genes to the explant;

b) incubating the transformed explant in a callus growth medium for a period of from about 15 to about 200 hours at a temperature of from 25° to about 35°C under a cycle of about 16 hours light and 8 hours dark to develop callus from the explants;

c) contacting the incubated explants with a callus growth medium containing an antibiotic toxic to *Agrobacterium* for a time sufficient to kill the *Agrobacterium*;

d) culturing the callus free of *Agrobacterium* on a callus growth medium;

e) contacting the resulting embryogenic callus with the antibiotic in a concentration sufficient to permit selection of callus resistant to the antibiotic; and

f) selecting transformed embryogenic callus.

39. The method of claim 38 further comprising the step of germinating the transformed callus and developing plantlets therefrom.

40. The method of claim 38 in which the transformed callus prior to contact with the callus growth medium in step c is rinsed in callus growth medium free of the antibiotic toxic to *Agrobacterium*.

41. The method of claim 38 wherein the cotton seedling explant is selected from hypocotyl, cotyledon and mixtures thereof.

42. The method of claim 38 wherein the callus growth medium is a Murashige and Skoog medium supplemented with about 1 to about 10 mg/liter naphthaleneacetic acid.

43. The method of claim 38 wherein the antibiotic toxic to *Agrobacterium* is cefotaxime.

44. A method of transforming cotton cells undergoing suspension culture on a callus growth medium which comprises, after a suspension subculture growth cycle:

a) recovering cells and any embryogenic callus from the callus growth medium;

b) resuspending the cells and embryogenic callus in a callus growth medium containing an *Agrobacterium* vector containing a chimeric gene that expresses a polypeptide having

substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein and a gene that confers resistance to an antibiotic on cotton cells while maintaining suspension growth conditions for a period of time sufficient to transform the suspended cells;

c) recovering the suspended cells from the callus growth medium containing the *Agrobacterium*;

d) treating the transformed cells and the embryogenic callus with an antibiotic toxic to *Agrobacterium* in sufficient concentration and for a time sufficient to kill the *Agrobacterium*;

e) contacting the cells and embryogenic callus with the antibiotic in order to select the transformed cells and embryogenic callus;

f) filtering the suspension to remove embryogenic callus greater than about 600 µm.

45. The method of claim 44 wherein steps d and e occur before step f.

46. The method of claim 44 wherein steps d and e occur after step f.

47. The method of claim 44 wherein step d occurs before step f and step e occurs after step f.

48. The method of claim 44 wherein step e occurs before step f and step d occurs after step f.

49. The method of claim 44 wherein the antibiotic of step d is cefotaxime.

50. The method of claim 44 wherein the suspension subculture growth cycle is from about 7 to about 14 days.

51. The method of claim 44 further comprising the step of developing the transformed cotton cells into plantlets.

52. Cotton plants transformed to express a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein and have resistance to the antibiotic hygromycin.

53. A method for protecting cotton plants against Dipteran and Lepidopteran insect damage comprising the expression of a *Bt* crystal protein or a protein having substantially the insect toxicity properties of a *Bt* crystal protein in the plant cells constituting the plant, in an amount sufficient to kill or to control the insect larvae.

54. A method according to claim 53 wherein the insect larvae are lepidopteran or dipteran larvae.

55. A method according to claim 54 wherein the insect larvae are lepidopteran larvae.

56. A method for killing or controlling Dipteran and Lepidopteran insect larvae by feeding them cotton plant cells containing chimeric genes that express an insecticidal amount of a toxin having substantially the insect toxicity properties of *Bt* crystal protein.

57. A method according to any one of claims 53 or 56, wherein the crystal protein is of the *Bt* variety *kurstaki* HD1.

58. A cotton cell comprising a chimeric gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein, exhibiting toxicity toward Dipteran and Lepidopteran insects substantially as herein described with reference to any one of Examples 11 to 32.

59. A cotton plant comprising a gene that expresses a polypeptide having substantially the insect toxicity properties of *Bacillus thuringiensis* crystal protein, exhibiting toxicity toward Dipteran and Lepidopteran insects substantially as hereinbefore described with reference to Examples 11 to 32.

60. A method of producing transformed, embryogenic cotton callus substantially as hereinbefore described with reference to Examples 11 to 32.

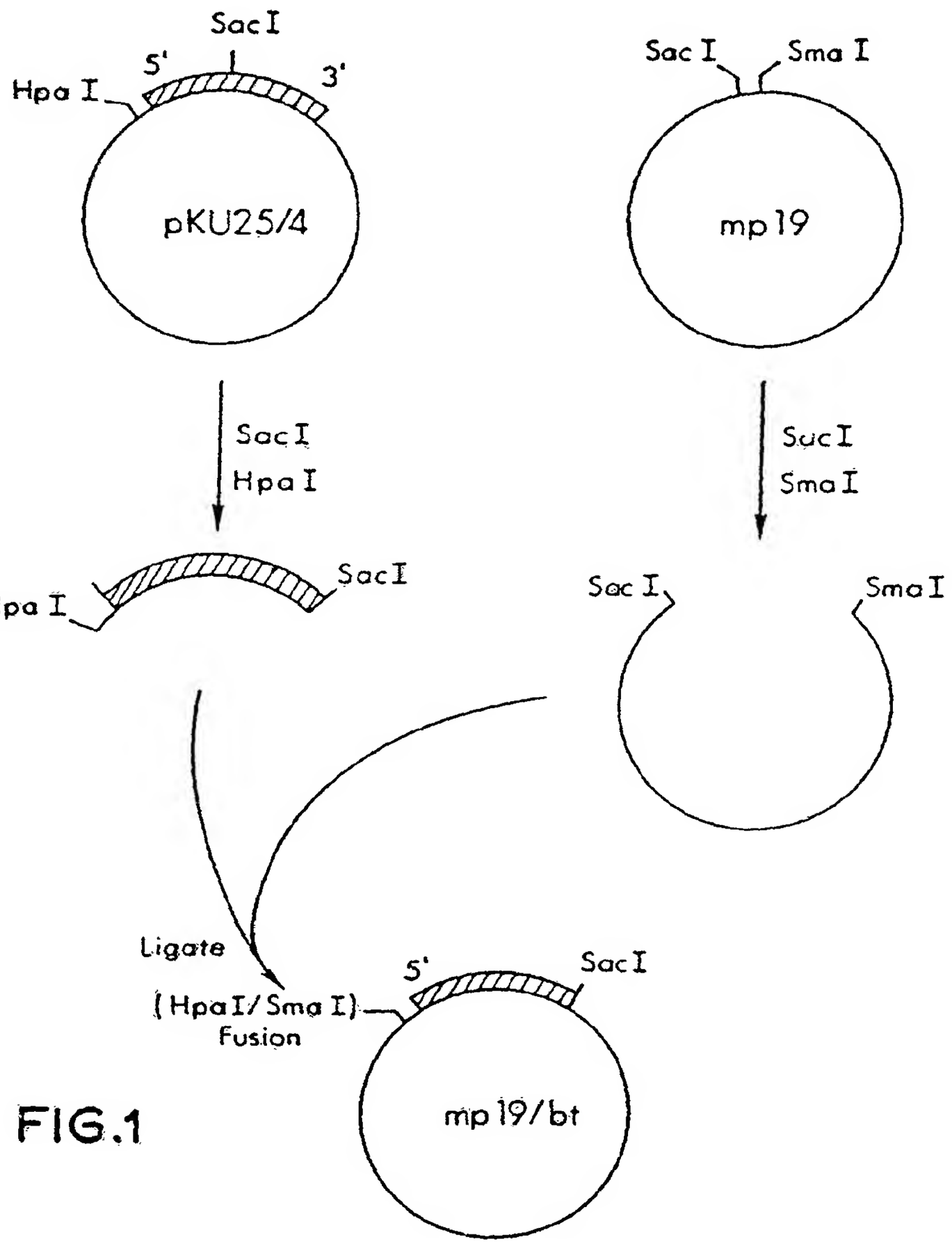
61. A method of transforming cotton cells undergoing suspension culture on a callus growth medium substantially as hereinbefore described with reference to Examples 11 to 32.

GSA/LMM/TCW/1160v

62. A method for killing or controlling Dipteran and Lepidopteran insect larvae comprising feeding said larvae an insecticidally effective amount of a cotton plant cell as defined in claim 58.

DATED this TWENTY-SECOND day of AUGUST 1991
Ciba-Geigy AG

Patent Attorneys for the Applicant
SPRUSON & FERGUSON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Ligate
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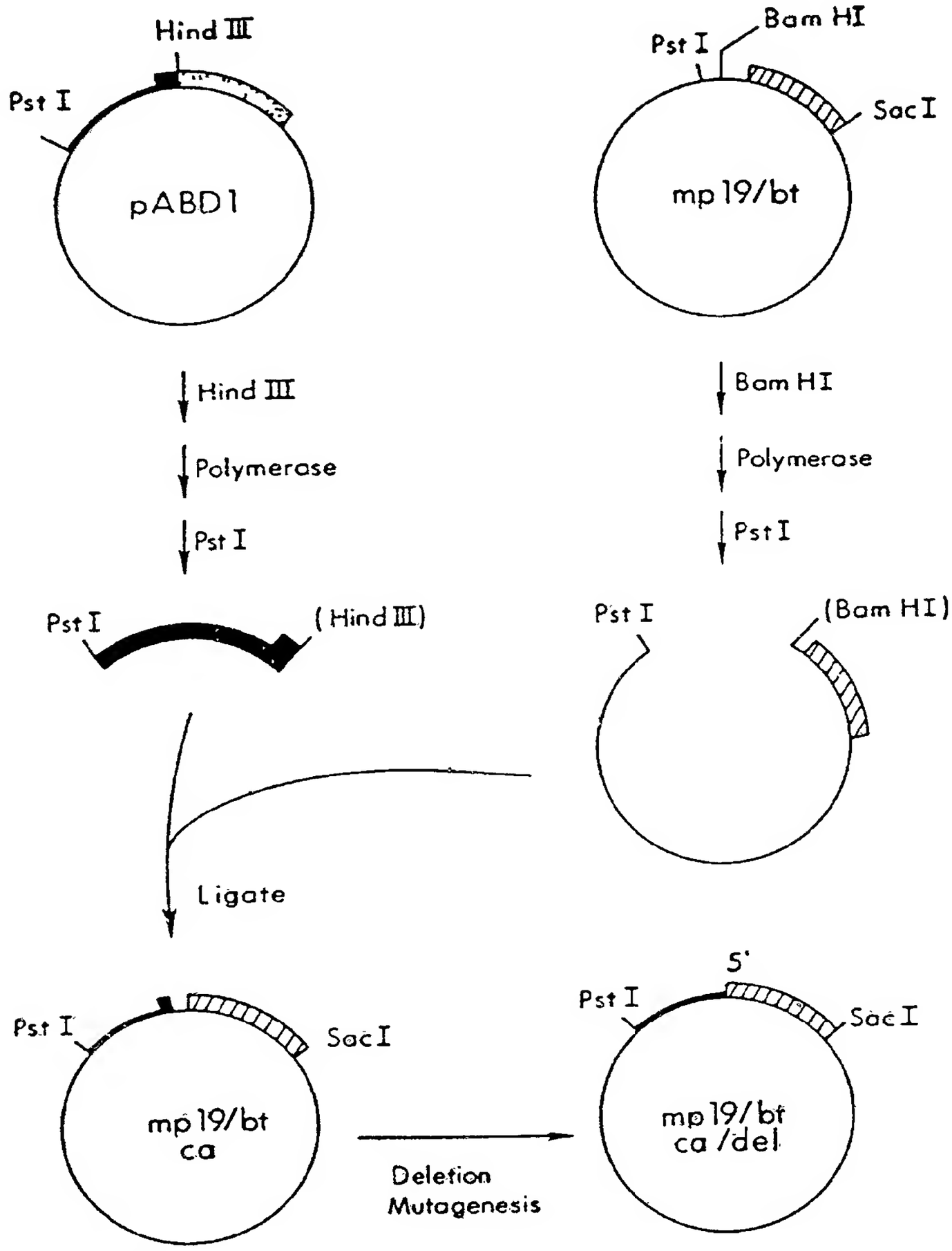
Sac I
(Hpa I/ Sma I)
Fusion
mp19/bt

FIG.1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ca
Deletion
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mp 19/bt
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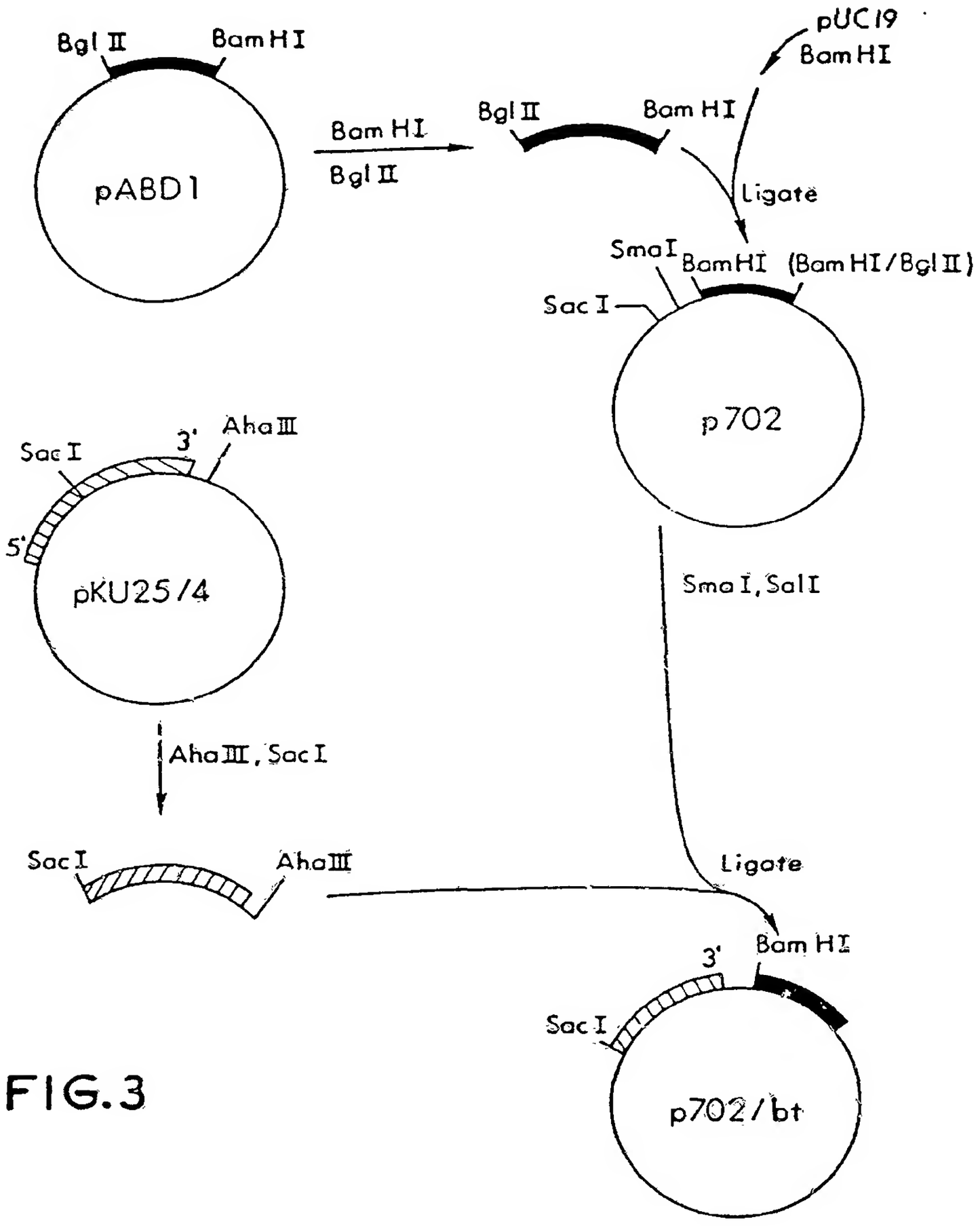
SacI

FIG. 2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Bgl II
Bgl II
Bam HI
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Ligate
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Sac I
p702
Sac I
3'
Aha III
5'
pKU25/4
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Aha III, Sac I
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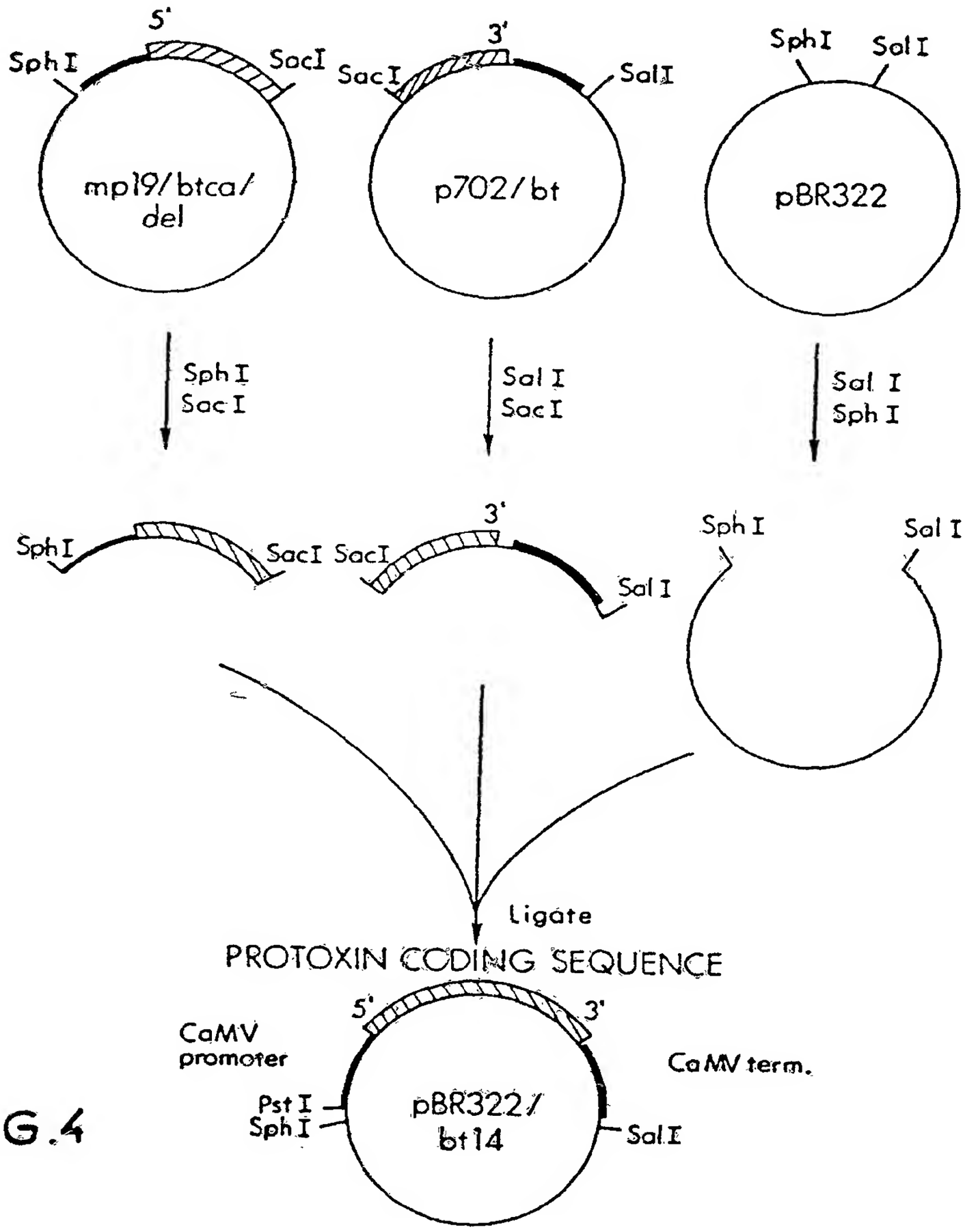
Aha III
Ligate
3'
Bam HI
Sac I
p702/bt

FIG.3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SacI
mp19/btca/del
3'
SacI
SalI
p702/bt
SphI
SalI
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SphI
SacI
SacI
SalI
SphI
SalI
Ligate
PROTOXIN CODING SEQUENCE
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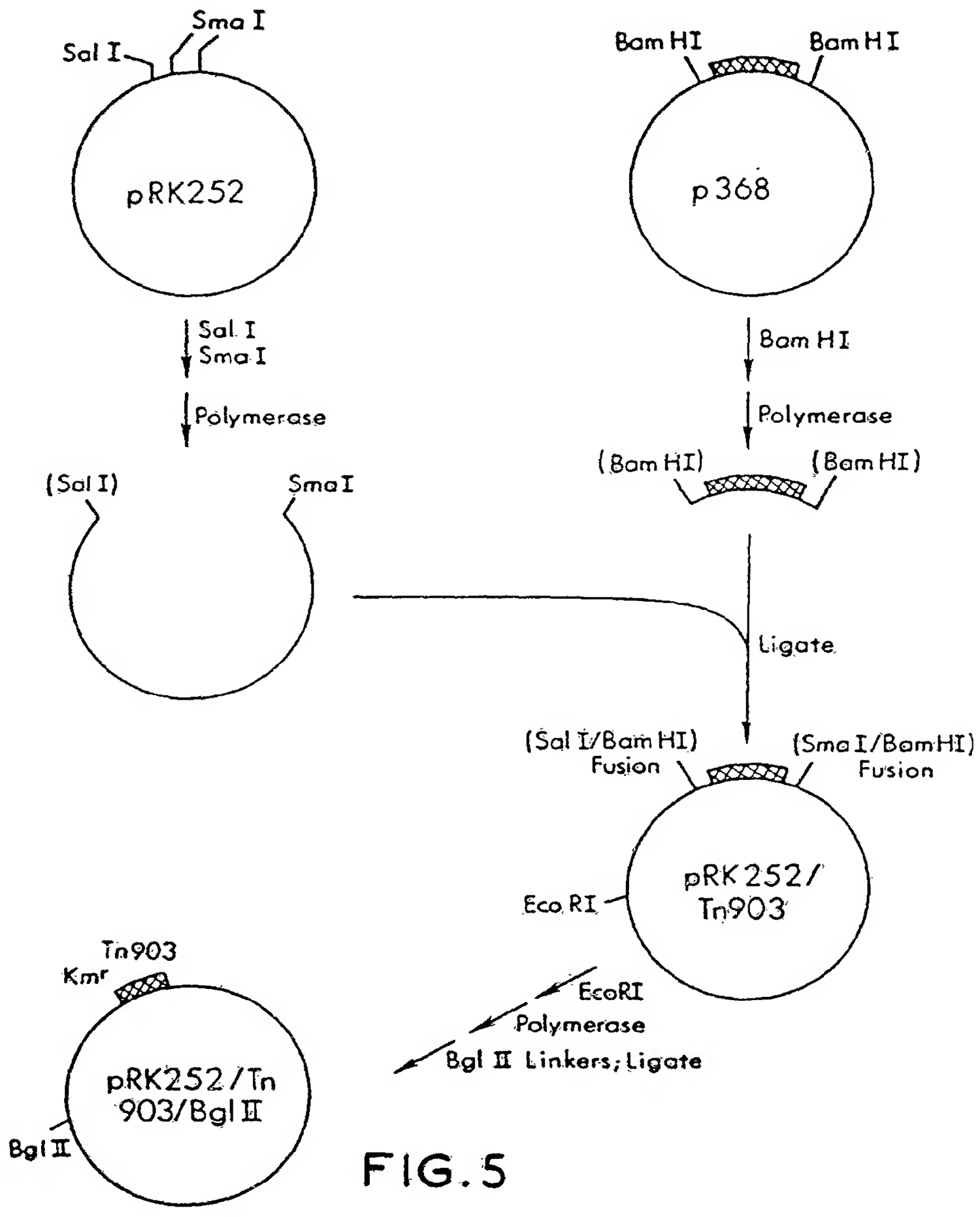
CaMV promoter
CaMV term.
PstI
SphI
pBR322/bt14
SalI

FIG.4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p368
Sal I
Sma I
Polymerase
Bam HI
Polymerase
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(Bam HI)
Ligate
(Sal I/Bam HI)
Fusion
(Sma I/Bam HI)
Fusion
pRK252/
Tn903
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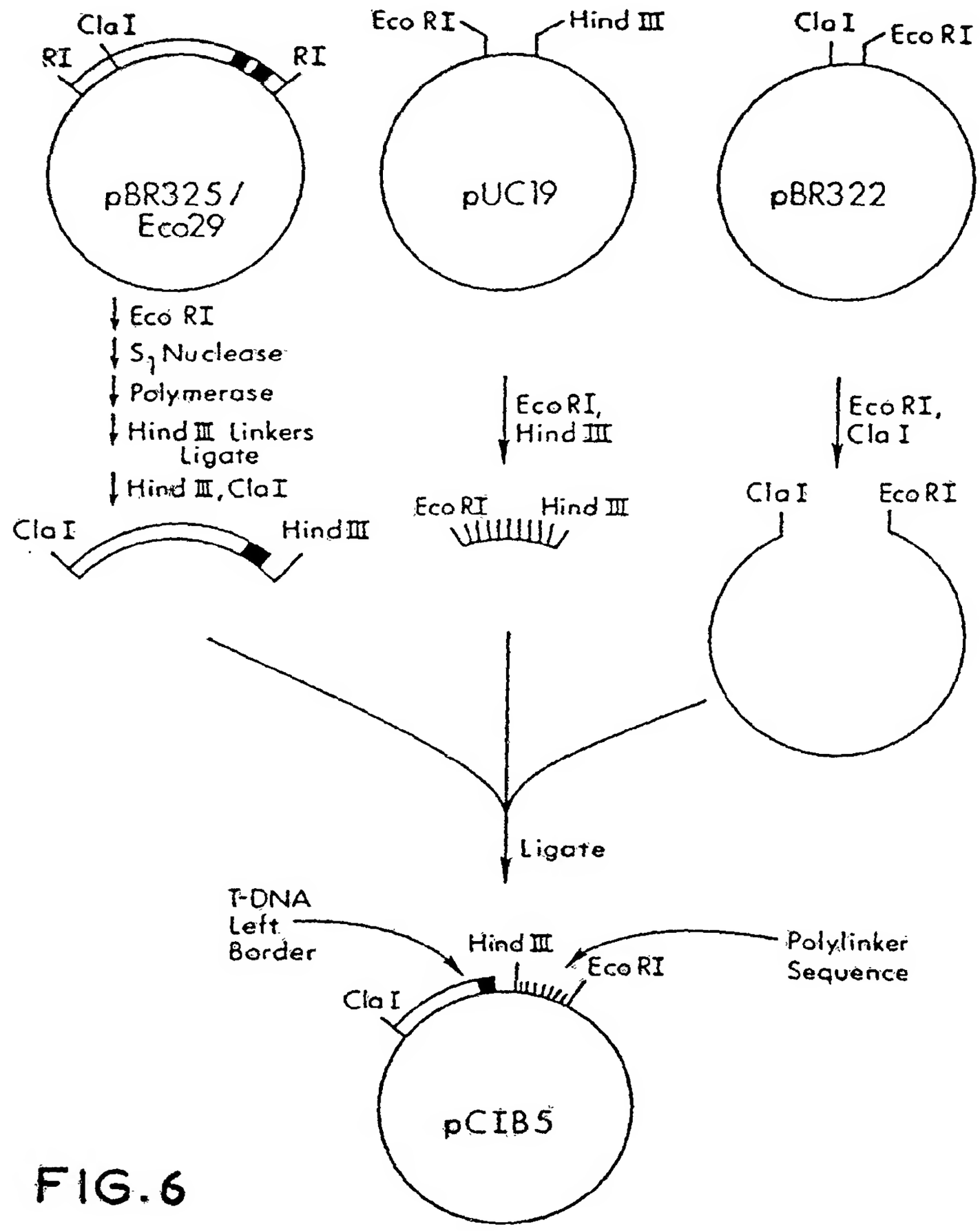
Polymerase
Bgl II Linkers; Ligate
Tn903
Kmr
pRK252/Tn
903/Bgl II
Bgl II

FIG. 5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RI
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Eco29
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pUC19
ClaI
Eco RI
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S1 Nuclease
Polymerase
Hind III Linkers
Ligate
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ClaI
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Hind III
Eco RI
Hind III
Eco RI,
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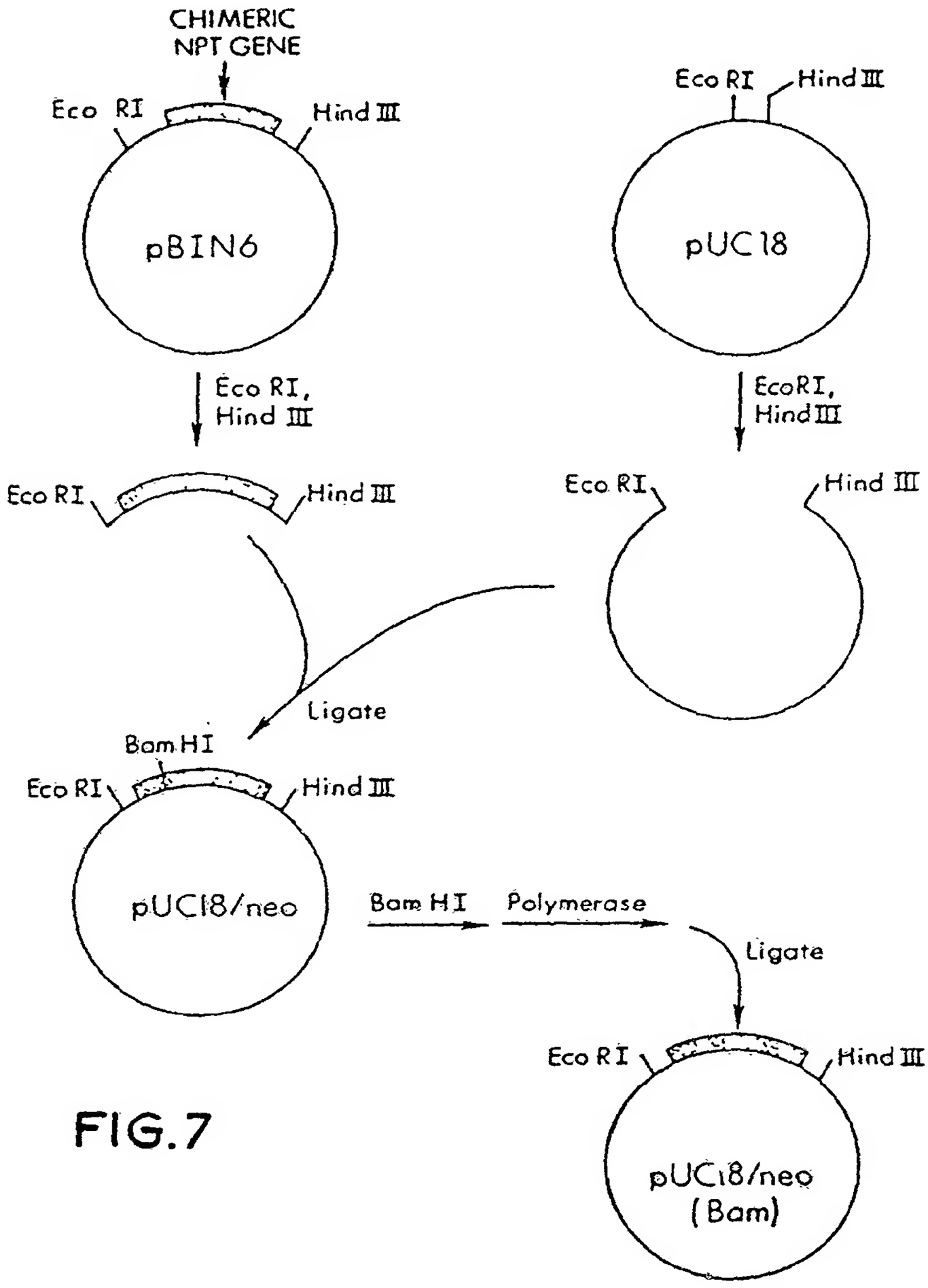
Eco RI
Polylinker
Sequence
ClaI
pCIB5

FIG. 6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Ligate
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Eco RI
Hind III
pUC18/neo
Bam HI
Polymerase
Ligate
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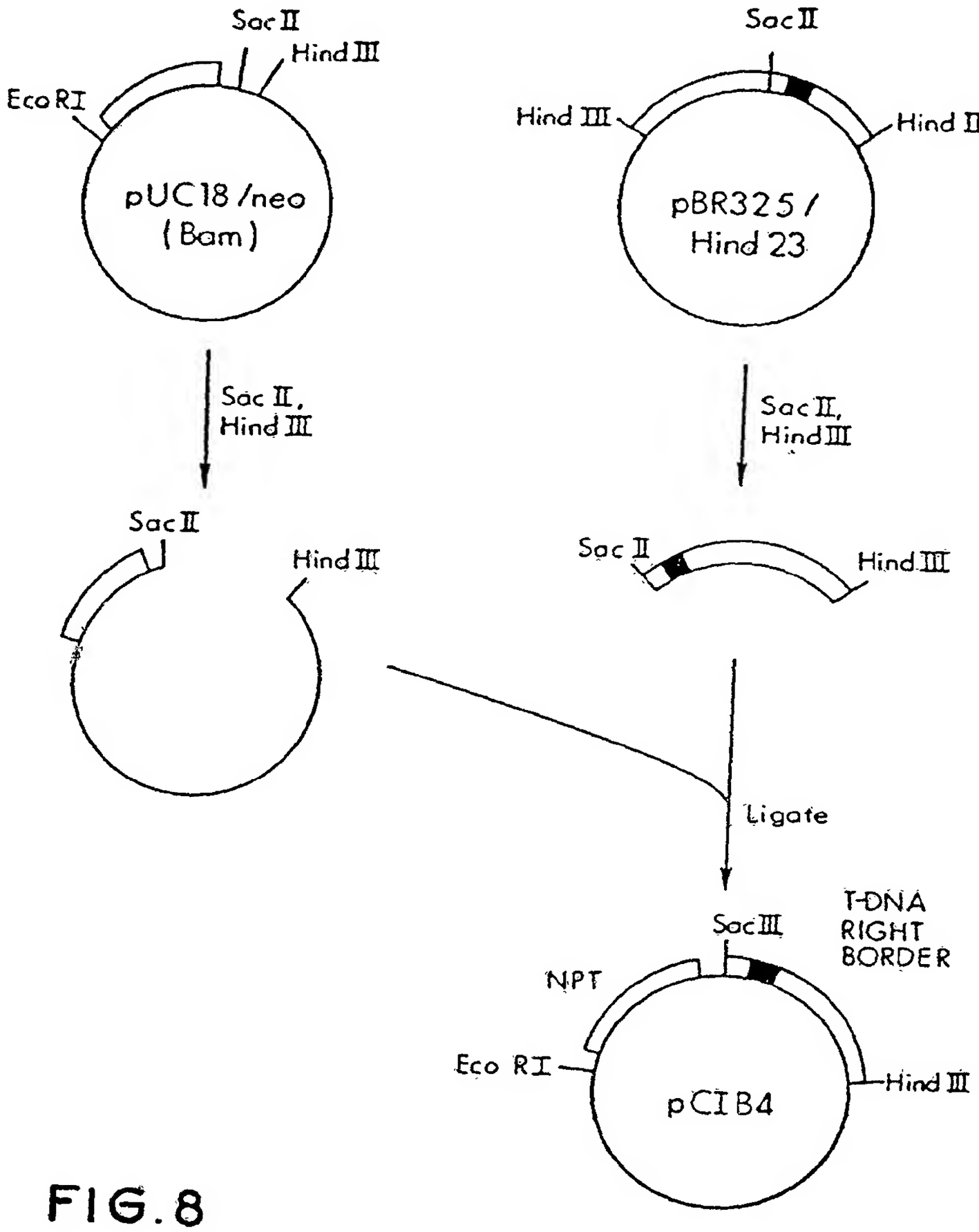
Eco RI
Hind III

FIG.7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NPT
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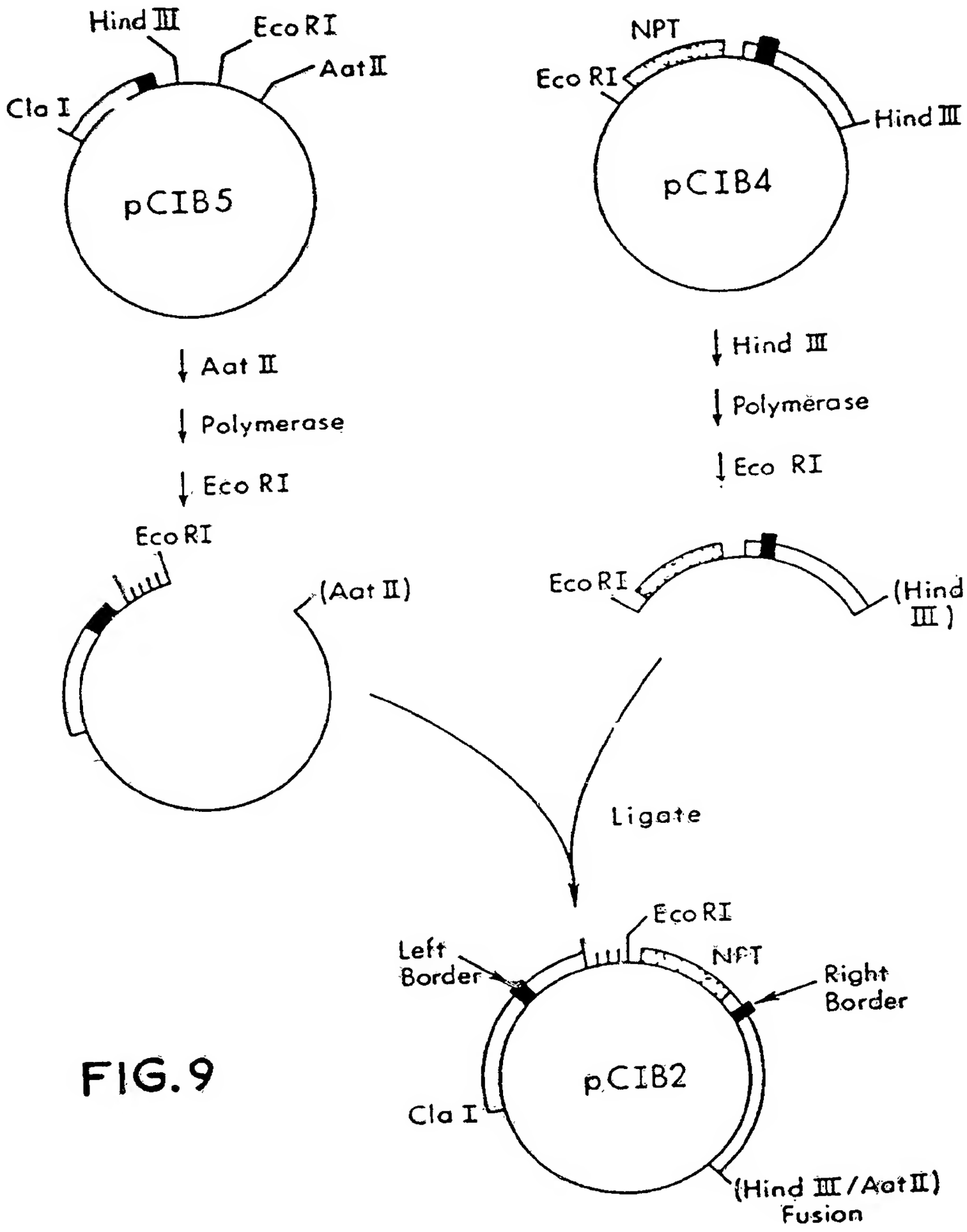
Hind III

FIG. 8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↓ EcoRI
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(Aat II)
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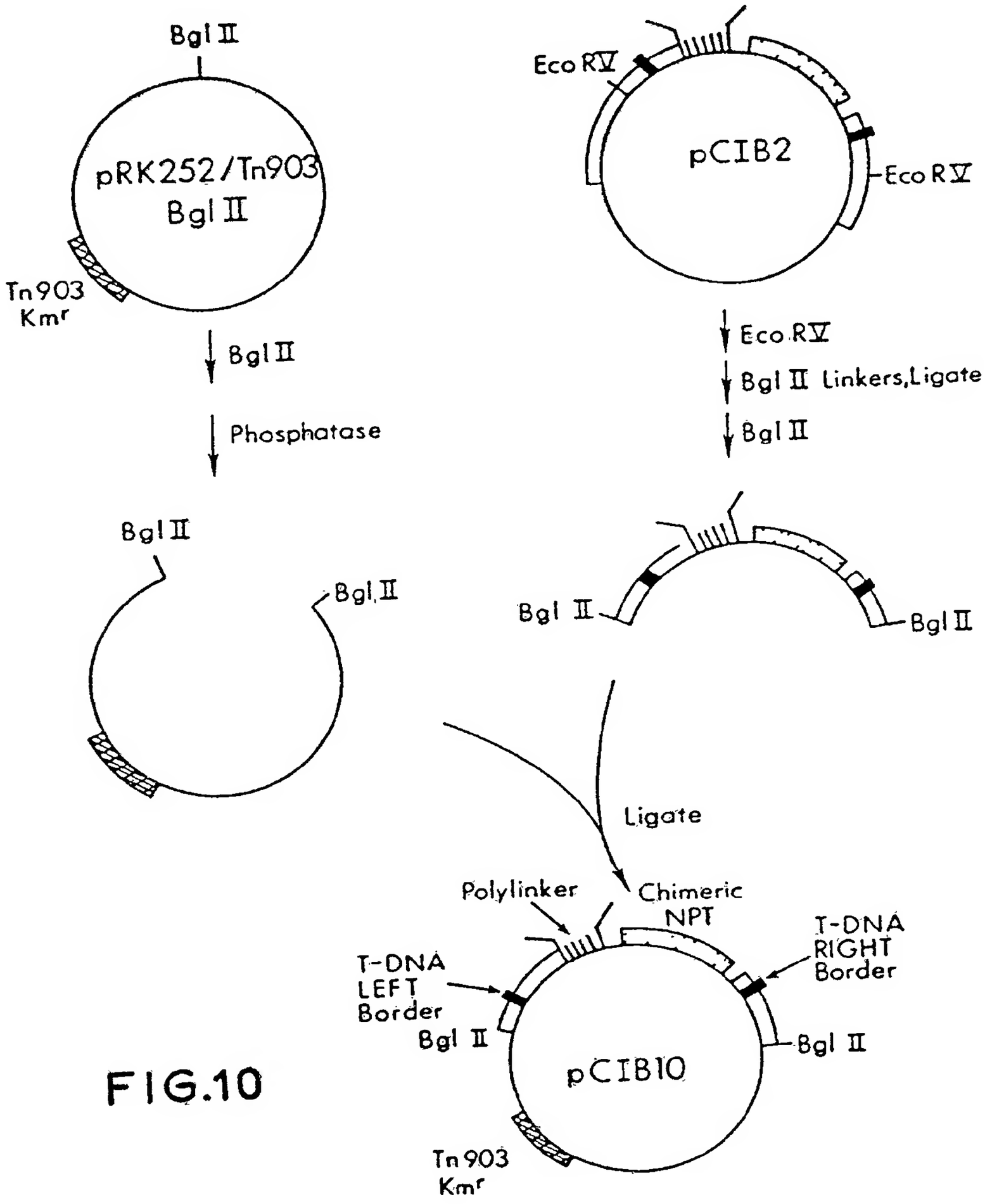
EcoRI
Hind III
pCIB4
↓ Hind III
↓ Polymerase
↓ EcoRI
EcoRI
(Hind III)
Ligate
EcoRI
Left Border
NPT
Right Border
ClaI
pCIB2
(Hind III/AatII) Fusion

FIG.9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Bgl II
Tn903
Kmr
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Phosphatase
Bgl II
Bgl II
Eco RV
pCIB2
Eco RV
Eco RV
Bgl II Linkers, Ligate
Bgl II
Bgl II
Bgl II
Ligate
Polylinker
Chimeric
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LEFT
Border
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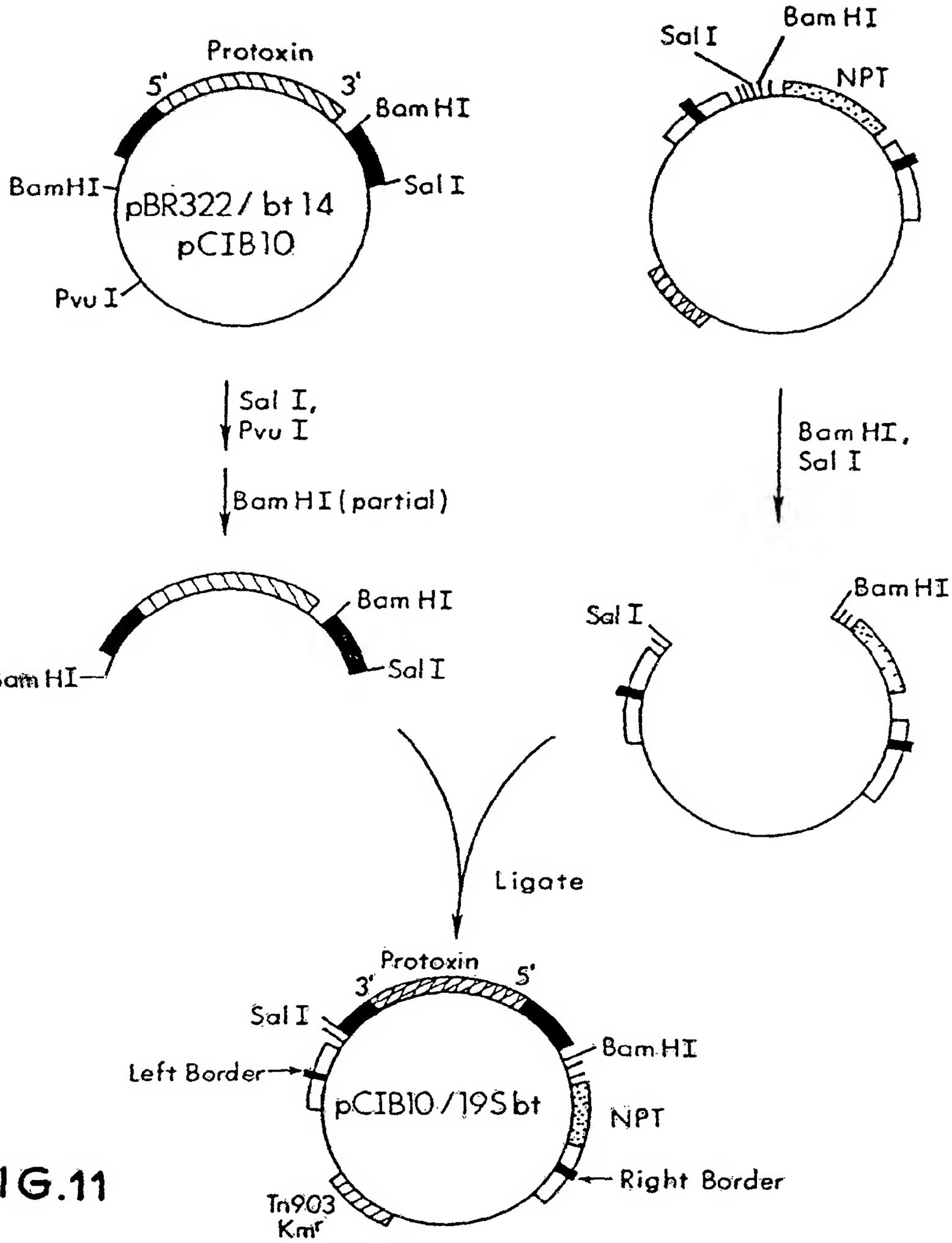
Tn903
Kmr

FIG.10


Protoxin
5'
3'
BamHI
BamHI
SalI
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pCIB10
PvuI
SalI,
PvuI
BamHI (partial)
BamHI
BamHI
SalI
SalI
BamHI
BamHI,
SalI
SalI
BamHI
NPT
Ligate
Protoxin
3'
5'
SalI
BamHI
Left Border
pCIB10/195bt
NPT
Right Border
Tn903
Kmr

FIG.11

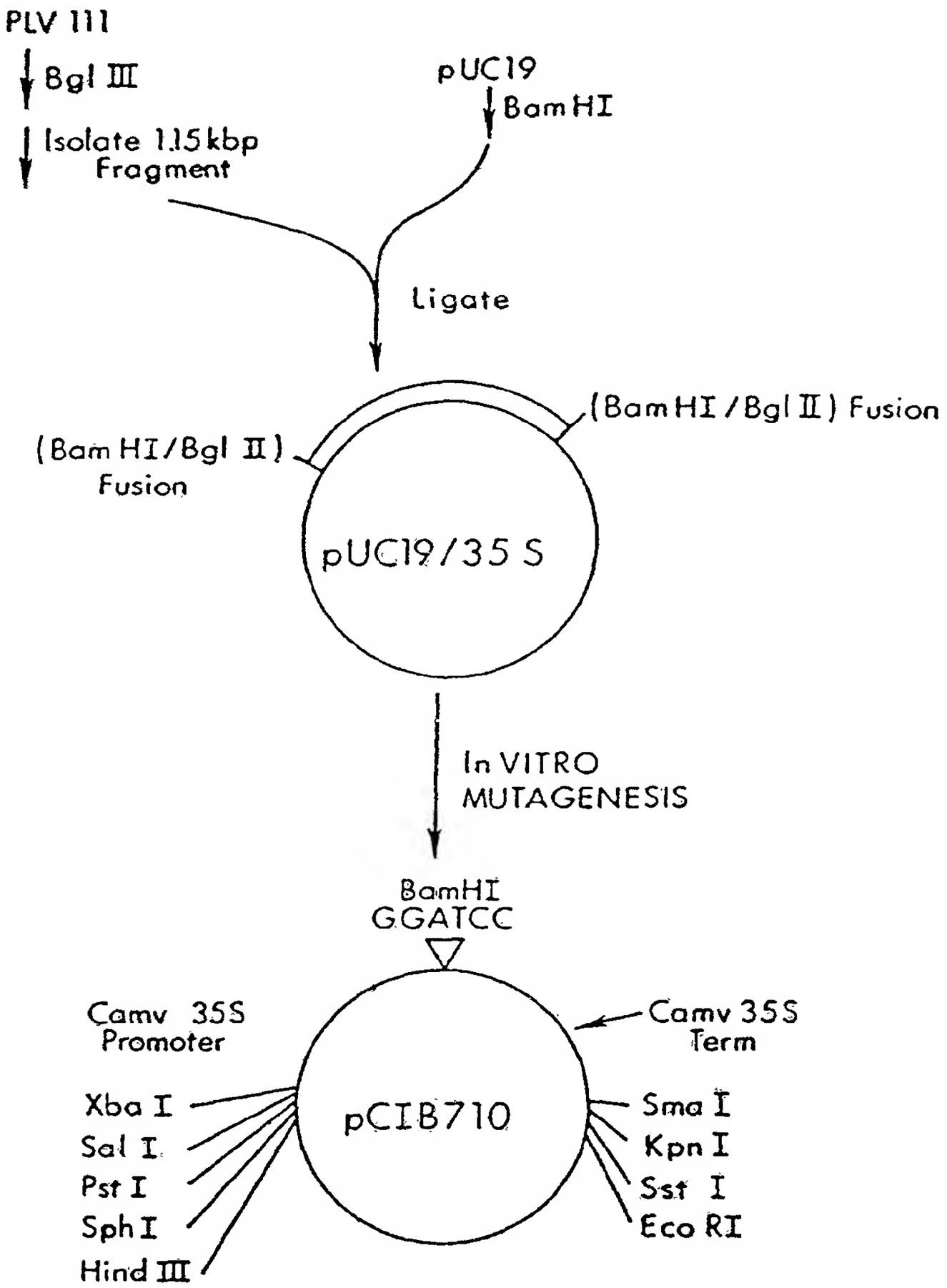
PLV 111
Bgl III
Isolate 1.15kbp Fragment
pUC19
BamHI
Ligate
(BamHI/Bgl II) Fusion
(BamHI/Bgl II) Fusion
pUC19/35 S
In VITRO MUTAGENESIS
BamHI
GGATCC
Camv 35S Promoter
Camv 35S Term
Xba I
Sal I
Pst I
Sph I
Hind III
pCIB710
Sma I
Kpn I
Sst I
Eco RI

FIG.12

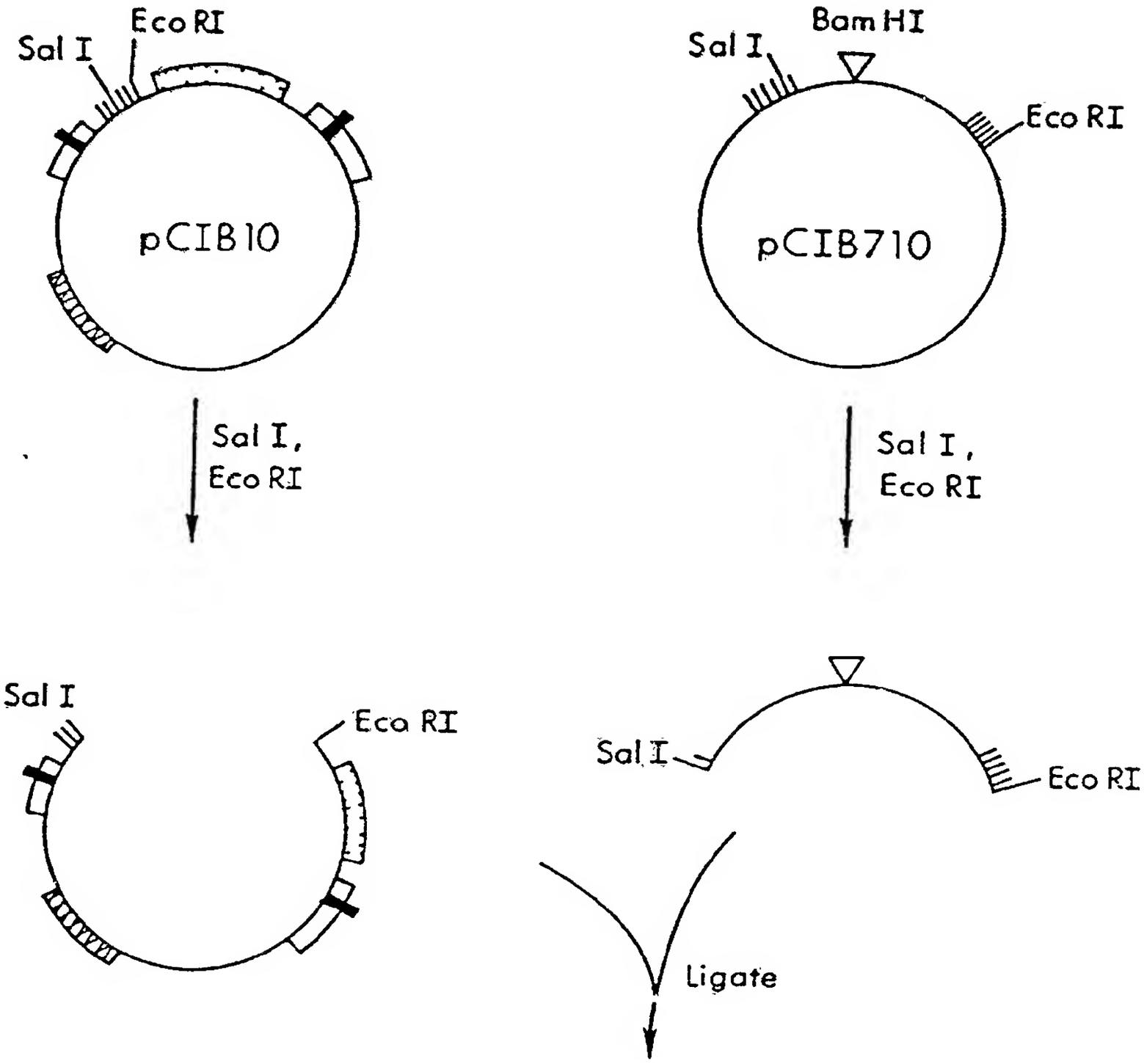
Sal I
Eco RI
pCIB10
Sal I
Bam HI
Eco RI
pCIB710
Sal I,
Eco RI
Sal I,
Eco RI
Sal I
Eco RI
Sal I
Eco RI
Ligate
35S
Promoter
Bam HI
35S Term
Left Border
NPT
pCIB10/710
Right Border
Tn 903
Kmr

FIG.13

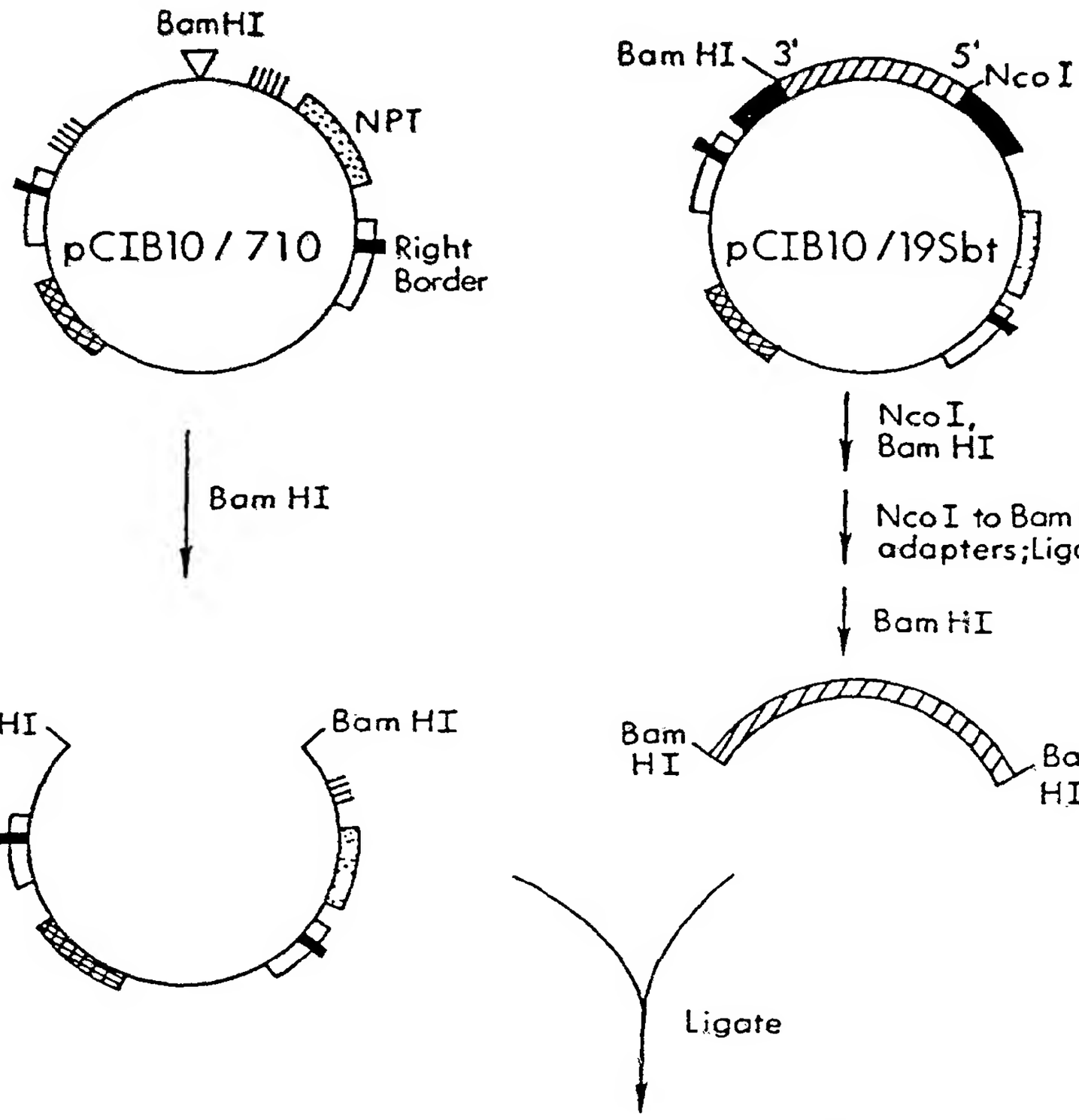
BamHI
NPT
pCIB10 / 710
Right Border
Bam HI
Bam HI
Bam HI
Bam HI
Bam HI 3' 5' Nco I
pCIB10 /19Sbt
Nco I, Bam HI
Nco I to Bam HI adapters; Ligate
Bam HI
Bam HI
Bam HI
Ligate
35S Promoter
Protoxin 3'
5'
35S Term
NPT
Left Border
pCIB10 / 35Sbt
Right Border
Tn903 Kmr

FIG.14

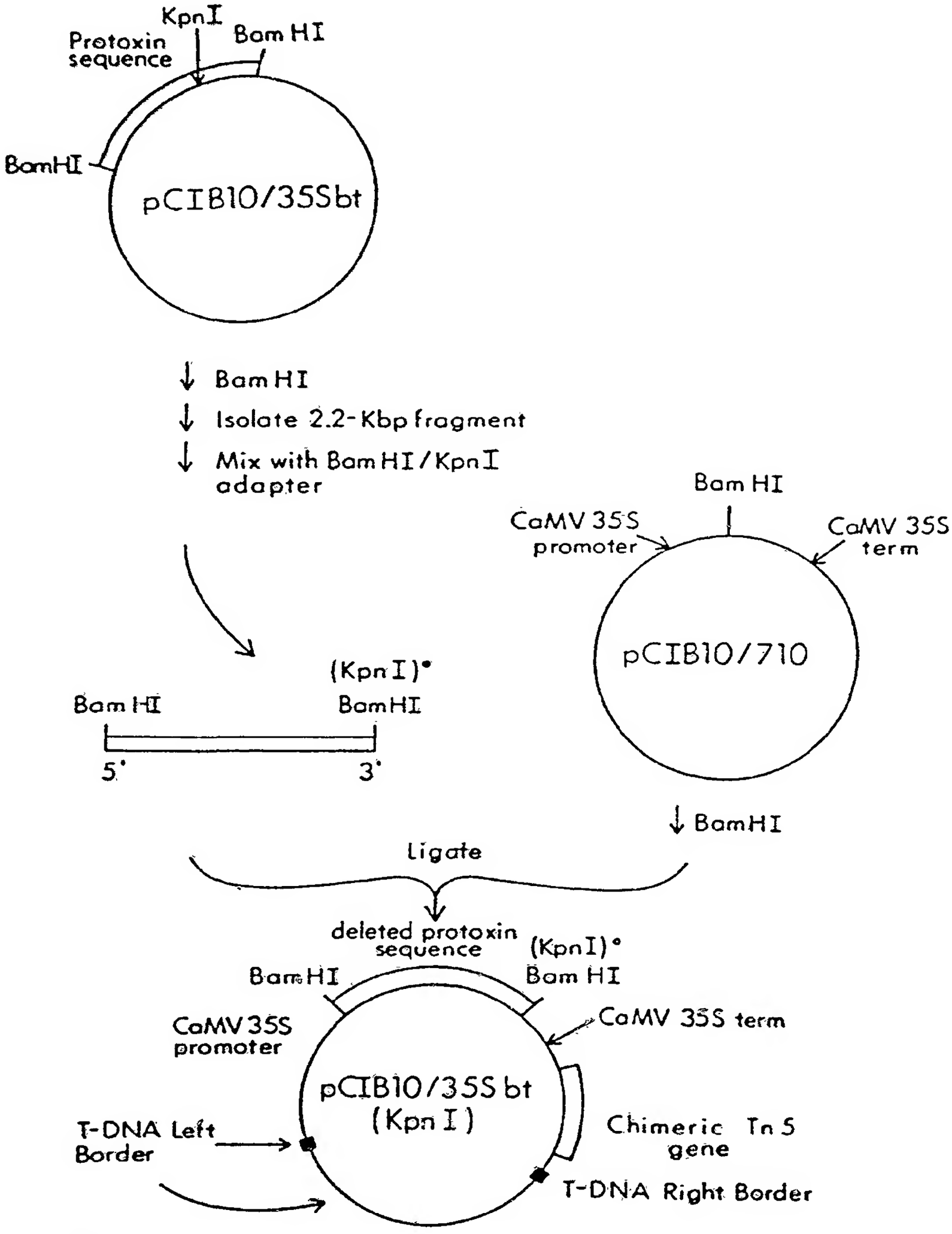
KpnI
Protoxin sequence
Bam HI
BamHI
pCIB10/35Sbt
BamHI
Isolate 2.2-Kbp fragment
Mix with BamHI/KpnI adapter
(KpnI)*
BamHI
BamHI
5'
3'
Bam HI
CaMV 35S promoter
CaMV 35S term
pCIB10/710
BamHI
Ligate
deleted protoxin sequence
(KpnI)*
BamHI
Bam HI
CaMV 35S promoter
CaMV 35S term
pCIB10/35S bt (Kpn I)
T-DNA Left Border
Chimeric Tn 5 gene
T-DNA Right Border

FIG.15

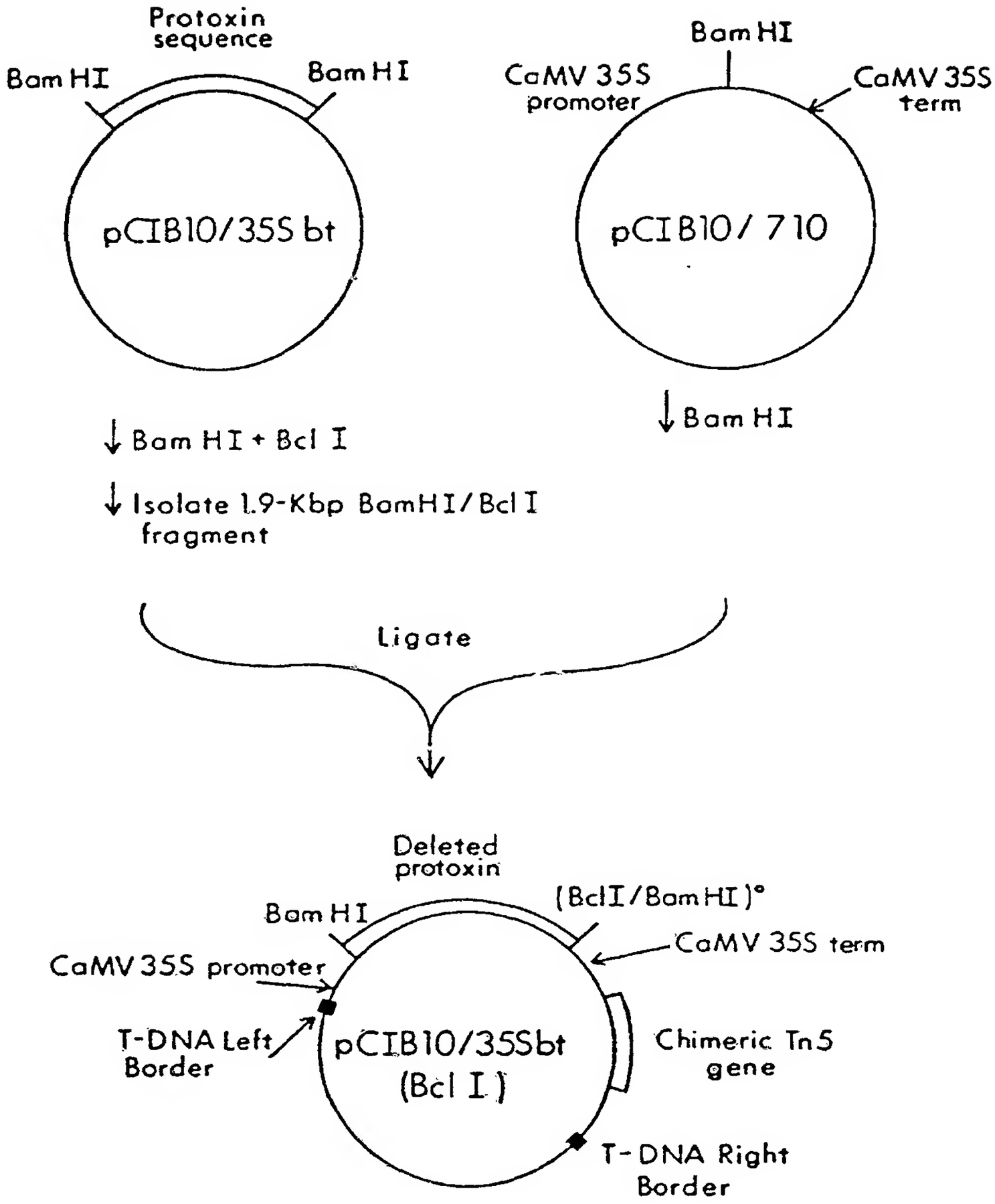
Protoxin sequence
Bam HI
Bam HI
pCIB10/35S bt
Bam HI
CaMV 35S promoter
CaMV 35S term
pCIB10/710
↓ Bam HI + Bcl I
↓ Isolate 1.9-Kbp BamHI/Bcl I fragment
↓ Bam HI
Ligate
Deleted protoxin
Bam HI
(BclI/BamHI)°
CaMV 35S term
CaMV 35S promoter
T-DNA Left Border
pCIB10/35Sbt (Bcl I)
Chimeric Tn5 gene
T-DNA Right Border

FIG.16

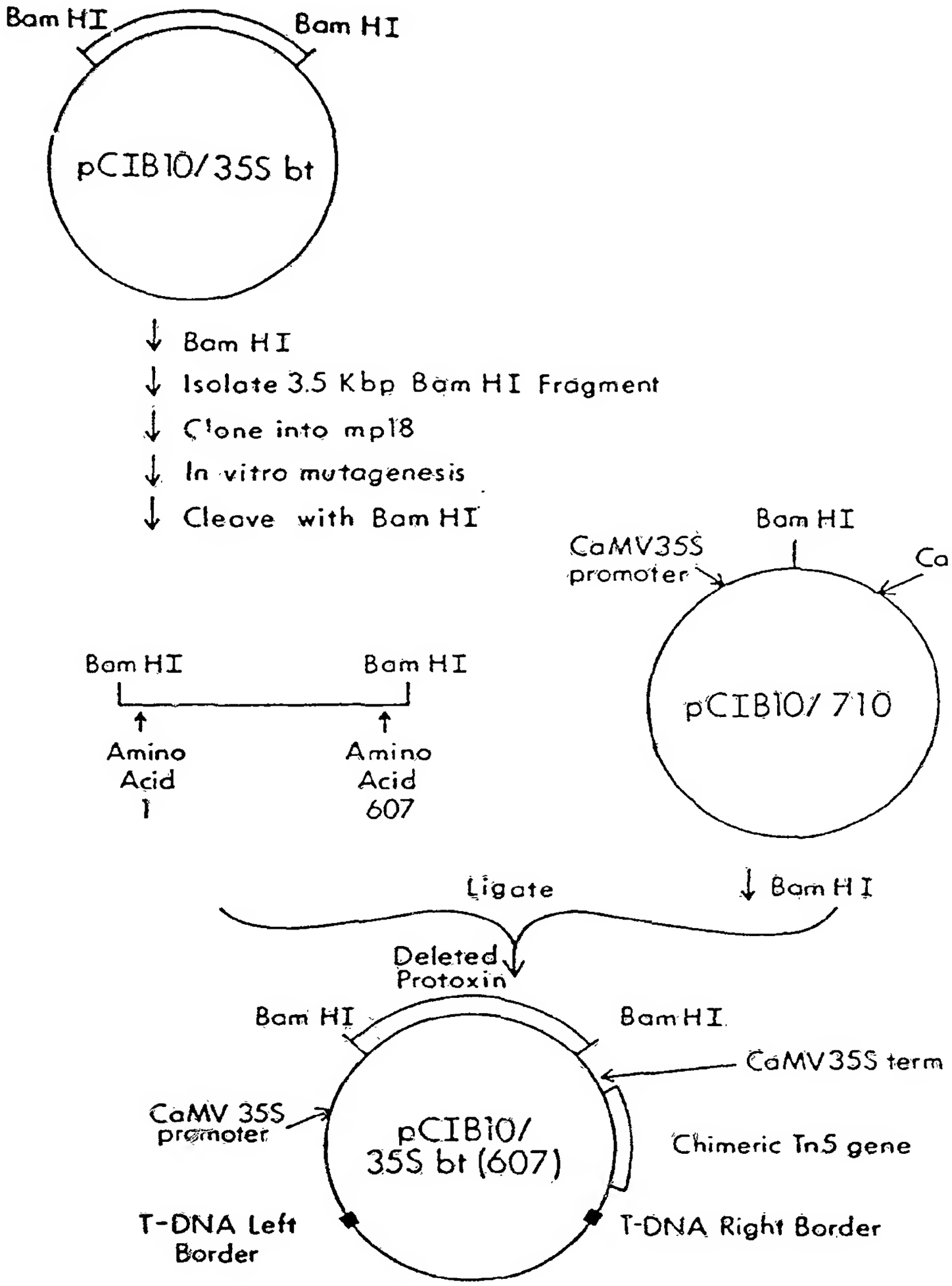
Bam HI
Bam HI
pCIB10/35S bt
Bam HI
Isolate 3.5 Kbp Bam HI Fragment
Clone into mp18
In vitro mutagenesis
Cleave with Bam HI
Bam HI
Bam HI
Amino Acid 1
Amino Acid 607
Bam HI
CaMV35S promoter
CaMV35S term
pCIB10/710
Ligate
Bam HI
Deleted Protoxin
Bam HI
Bam HI
CaMV35S term
CaMV 35S promoter
pCIB10/ 35S bt (607)
Chimeric TnS gene
T-DNA Left Border
T-DNA Right Border

FIG.17

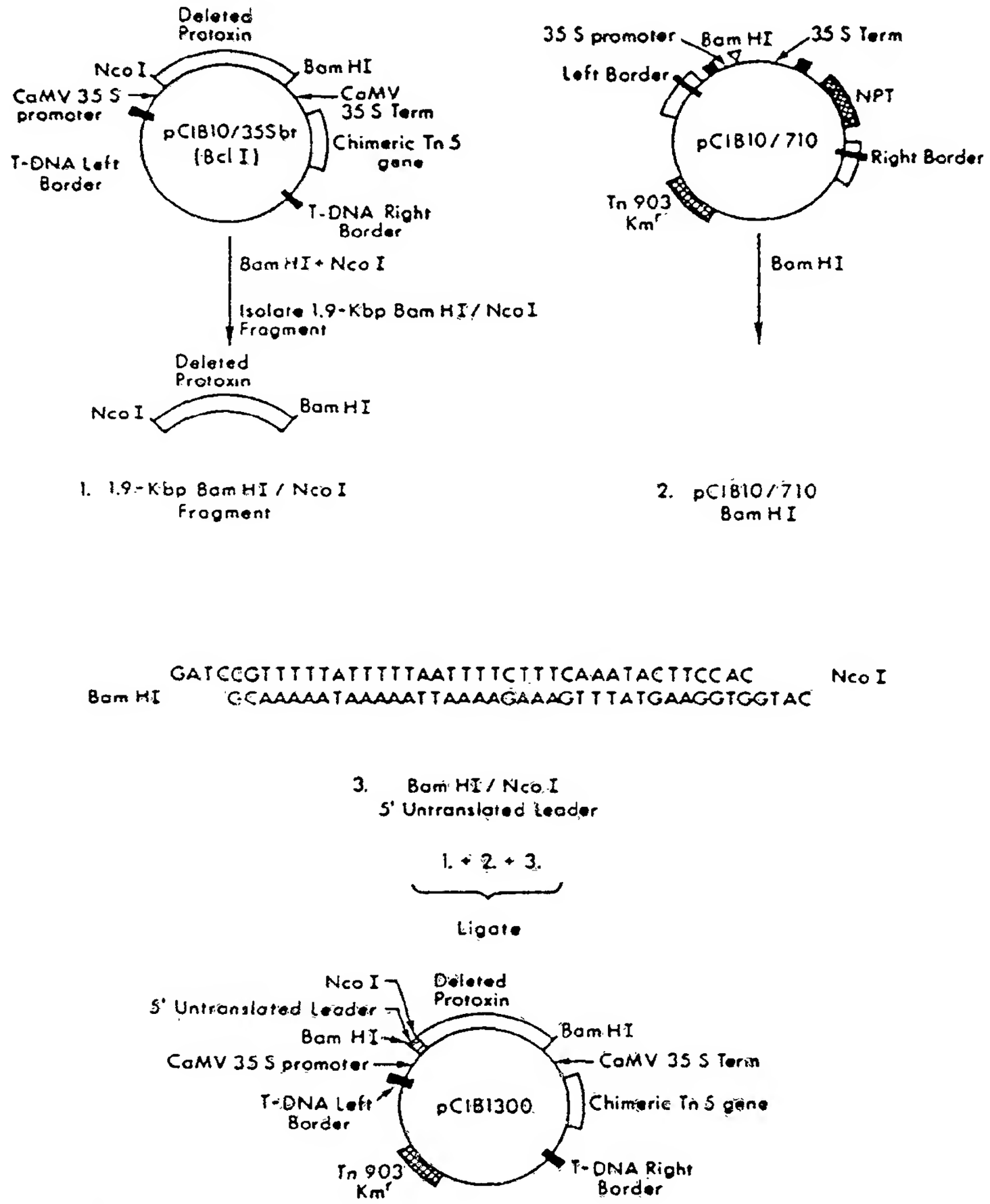
Deleted Protoxin
Nco I
Bam HI
CaMV 35 S promoter
CaMV 35 S Term
pCIB10/35Sbt (Bcl I)
Chimeric Tn 5 gene
T-DNA Left Border
T-DNA Right Border
Bam HI + Nco I
Isolate 1.9-Kbp Bam HI / Nco I Fragment
Deleted Protoxin
Nco I
Bam HI
35 S promoter
Bam HI
35 S Term
Left Border
NPT
pCIB10/710
Right Border
Tn 903 Km^r
Bam HI
1. 1.9-Kbp Bam HI / Nco I Fragment
2. pCIB10/710 Bam HI
GATCCGTTTTTATTTTTAATTTTCTTTCAAATACTTCCAC
Bam HI
CCAAAAATAAAAATTAAAAGAAAGTTTATGAAGGTGGTAC
Nco I
3. Bam HI / Nco I 5' Untranslated Leader
1. + 2. + 3.
Ligate
Nco I
Deleted Protoxin
5' Untranslated Leader
Bam HI
Bam HI
CaMV 35 S promoter
CaMV 35 S Term
T-DNA Left Border
pCIB1300
Chimeric Tn 5 gene
Tn 903 Km^r
T-DNA Right Border

FIG.19

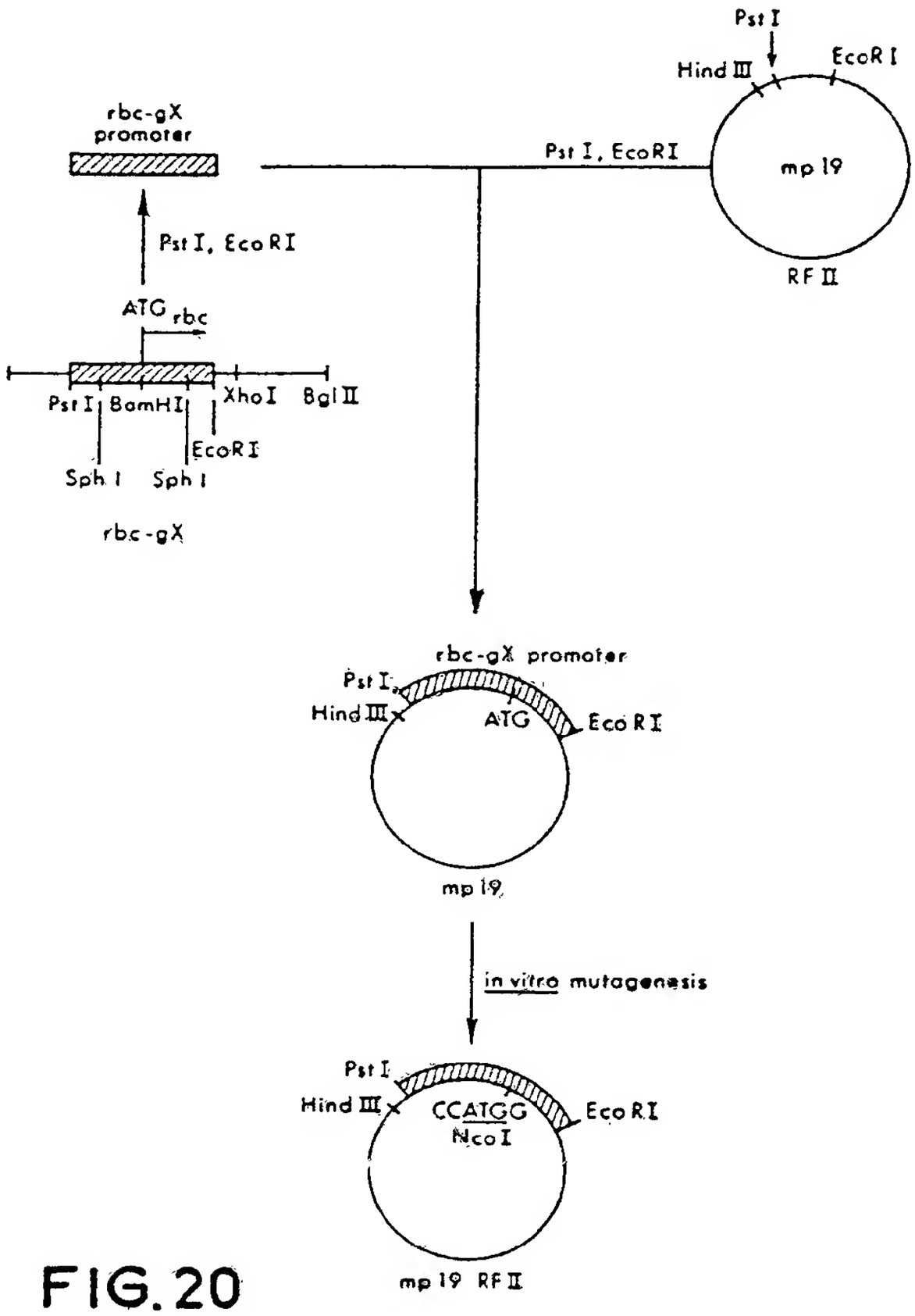
Pst I
Hind III
EcoR I
rbc-gX promoter
Pst I, EcoRI
mp 19
RF II
Pst I, Eco RI
ATG rbc
Pst I
BamHI
XhoI
BglII
EcoRI
Sph I
Sph I
rbc-gX
rbc-gX promoter
Pst I
Hind III
ATG
Eco R I
mp 19
in vitro mutagenesis
Pst I
Hind III
CCATGG
NcoI
Eco R I
mp 19 RF II

FIG. 20

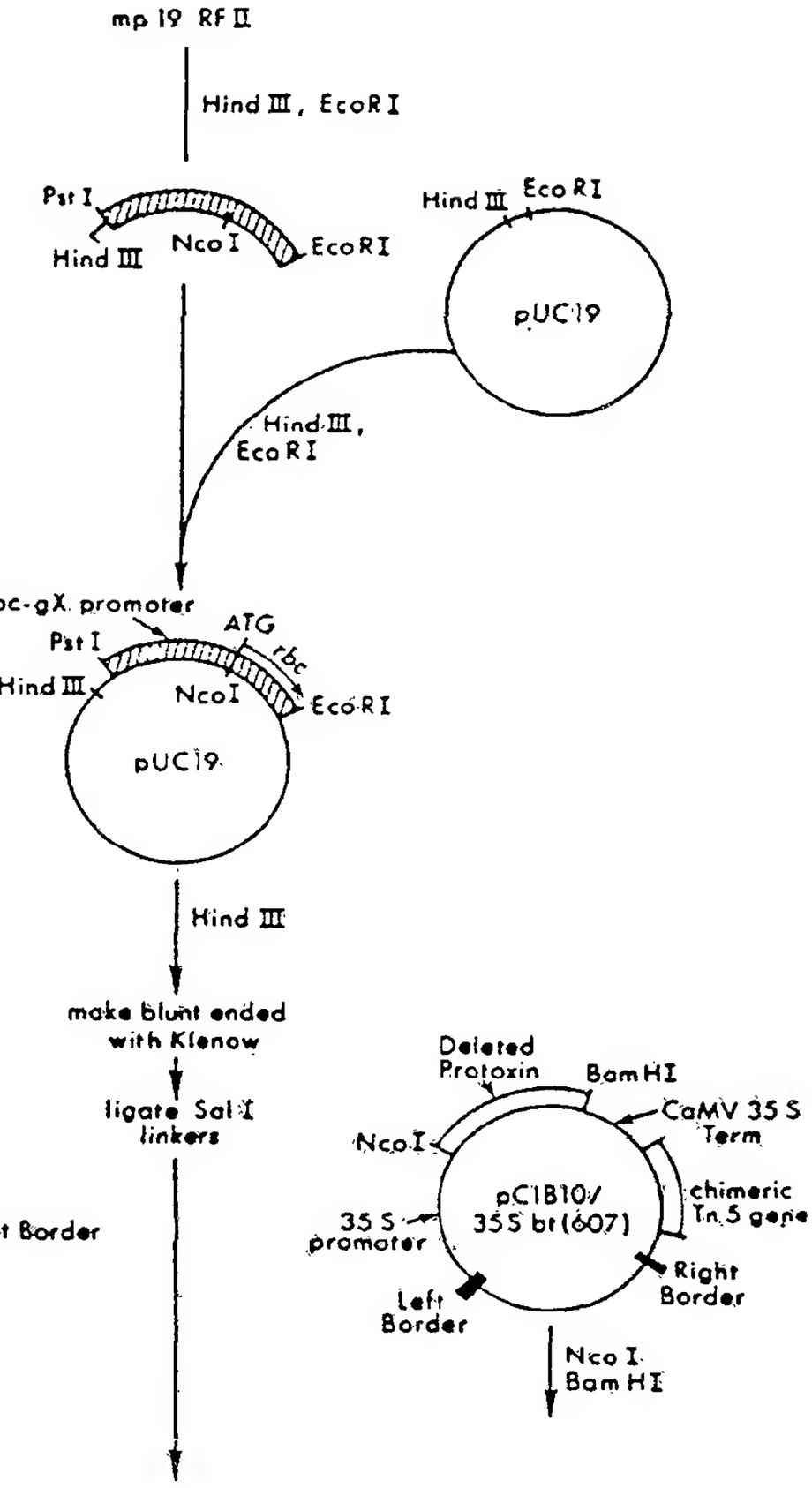
mp 19 RF II
Hind III, EcoR I
Pst I
Hind III
Nco I
EcoRI
Hind III
Eco RI
pUC19
Hind III,
Eco RI
rbc-gX promoter
ATG
rbc
Pst I
Hind III
Nco I
Eco RI
pUC19
Hind III
make blunt ended
with Klenow
ligate Sal I
linkers
35 S
Promoter
Bam HI
35 S Term
Left
Border
Sal I
pCIB10/710
NPT
Right Border
Tn 903
Km
Bam HI
Sal I
Deleted
Protoxin
Bam HI
CaMV 35 S
Term
Nco I
pCIB10/
35S bt(607)
chimeric
Tn 5 gene
35 S
promoter
Left
Border
Right
Border
Nco I
Bam HI

FIG.21

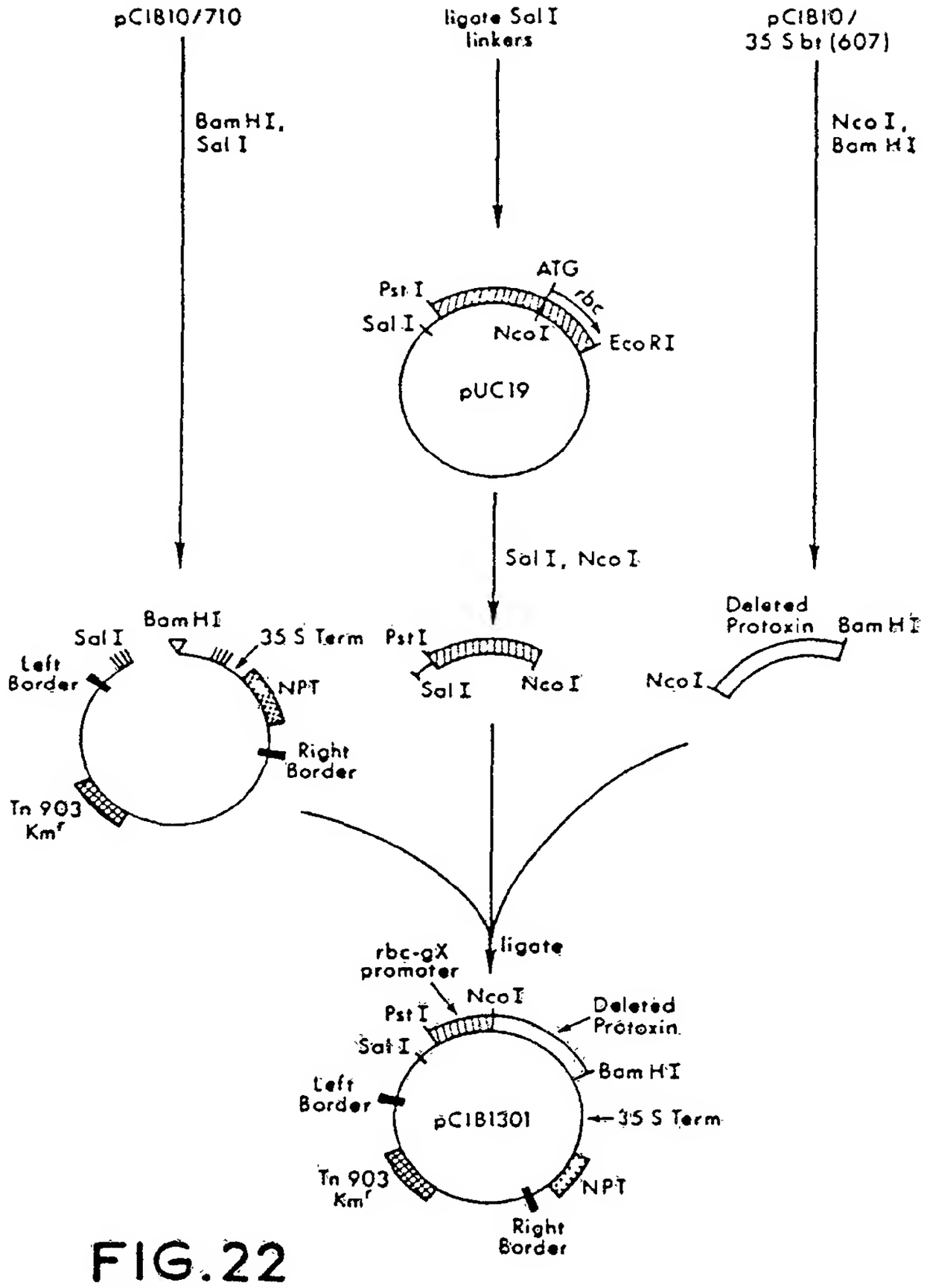
pC1810/710
BamHI,
Sal I
ligate Sal I
linkers
pC1810/
35 S bt (607)
Nco I,
Bam HI
ATG
rbc
Pst I
Sal I
Nco I
Eco RI
pUC19
Sal I, Nco I
BamHI
Sal I
35 S Term
Left
Border
NPT
Right
Border
Tn 903
Km^r
Pst I
Sal I
Nco I
Deleted
Protoxin
BamHI
Nco I
ligate
rbc-gX
promoter
Nco I
Pst I
Sal I
Deleted
Protoxin
Bam HI
Left
Border
pC1B1301
35 S Term
Tn 903
Km^r
NPT
Right
Border

FIG.22

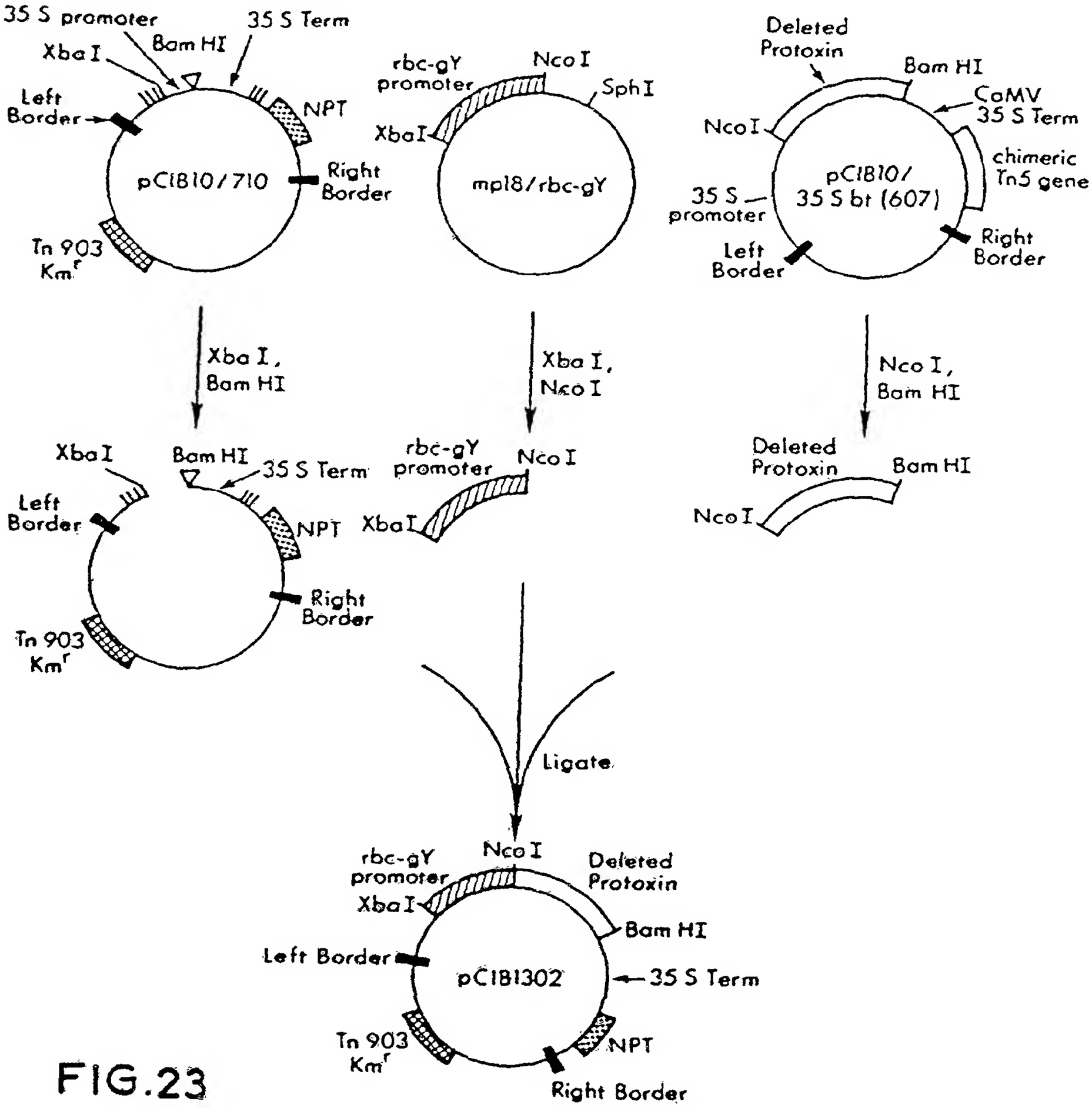
35 S promoter
Xba I
Bam HI
35 S Term
Left Border
NPT
pCIB10/710
Right Border
Tn 903 Km^r
rbc-gY promoter
Nco I
Sph I
Xba I
mp18/rbc-gY
Deleted Protoxin
Bam HI
CaMV 35 S Term
Nco I
chimeric Tn5 gene
pCIB10/ 35 S bt (607)
35 S promoter
Right Border
Left Border
Xba I, Bam HI
Xba I, Nco I
Nco I, Bam HI
Xba I
Bam HI
35 S Term
Left Border
NPT
Right Border
Tn 903 Km^r
rbc-gY promoter
Nco I
Xba I
Deleted Protoxin
Nco I
Deleted Protoxin
Bam HI
Ligate
rbc-gY promoter
Nco I
Deleted Protoxin
Xba I
Bam HI
Left Border
pCIB1302
35 S Term
Tn 903 Km^r
NPT
Right Border

FIG.23

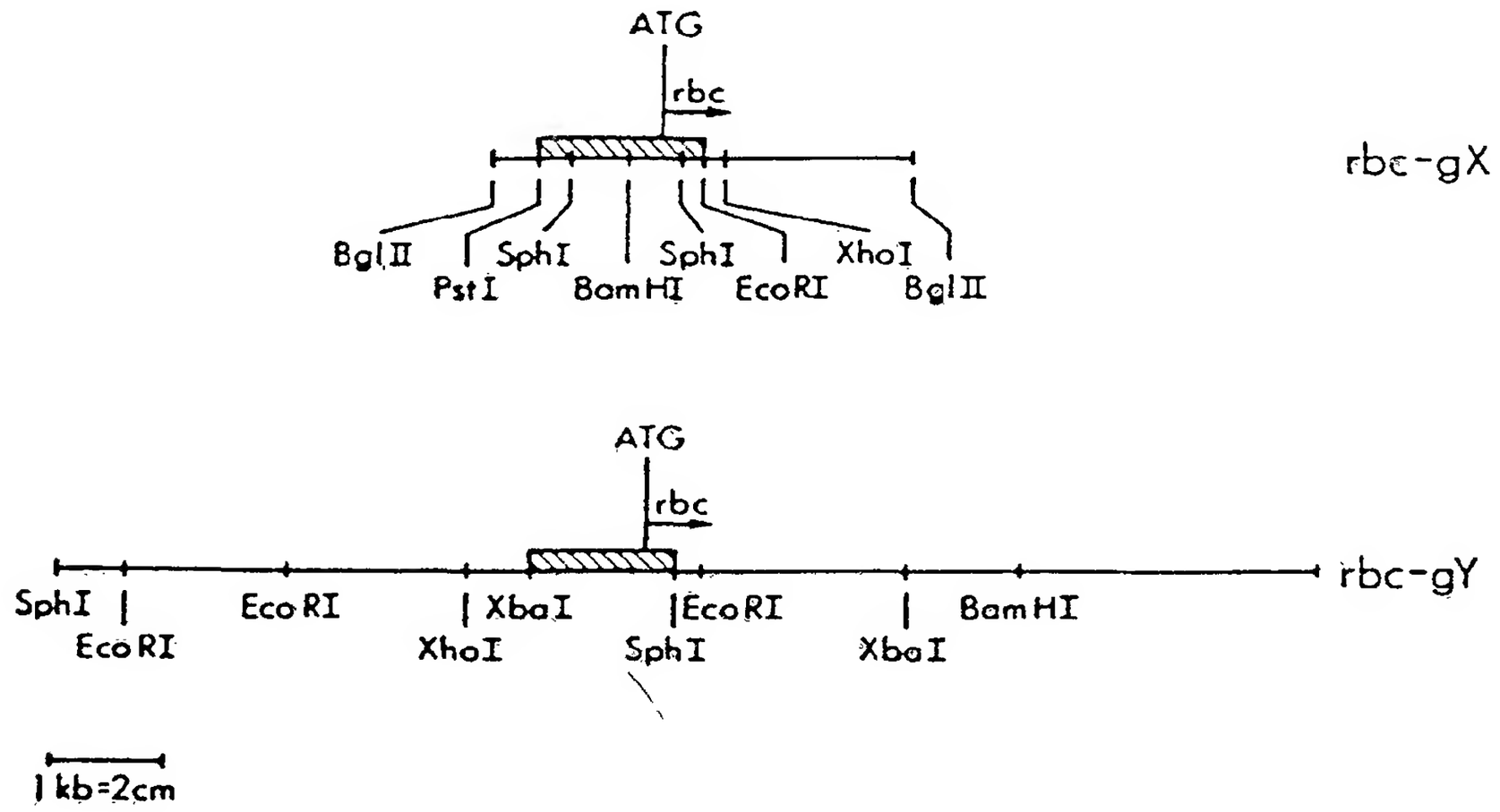
ATG
rbc
rbc-gX
BglII
PstI
SphI
BamHI
SphI
EcoRI
XhoI
BglII
ATG
rbc
rbc-gY
SphI
EcoRI
EcoRI
XhoI
XbaI
SphI
EcoRI
XbaI
BamHI
1 kb=2cm

FIG. 24

NUCLEOTIDE AND AMINO-ACID SEQUENCES OF RBC-GY

TRANSIT-PETIDE

```
    CTACTAGCAATGGCTTCCTCAATGATCTCATCGGCTACCATTGCCACTGCCTCTCCGGCA
1   ---------+---------+---------+---------+---------+---------+ 60
    GATGATCGTTACCGAAGGAGTTACTAGAGTAGCCGATGGTAACGGTGACGGAGAGGCCGT

      L   L   A   M   A   S   S   M   I   S   S   A   T   I   A   T   A   S   P   A   -

    CAGGCTAACATGGTCGCTCCTTTCACCGGCCTCAAGTCTGCCTCTGCTTTCCCAGTCATC
61  ---------+---------+---------+---------+---------+---------+ 120
    GTCCGATTGTACCAGCGAGGAAAGTGGCCGGAGTTCAGACGGAGACGAAAGGGTCAGTAG

      Q   A   N   M   V   A   P   F   T   G   L   K   S   A   S   A   F   P   V   I   -

    AGGAAGGCCAACAACGACATTACTTCTCTCGCAAGCAATGGCGGCAGAGTGCAATGC
121 ---------+---------+---------+---------+---------+---------
    TCCTTCCGGTTGTTGCTGTAATGAAGAGAGCGTTCGTTACCGCCGTCTCACGTTACG

      R   K   A   N   N   D   I   T   S   L   A   S   N   G   G   R   V   Q   C
```

FIG. 25

NUCLEOTIDE AND AMINO-ACID SEQUENCES OF RBC-GX

TRANSIT-PEPTIDE

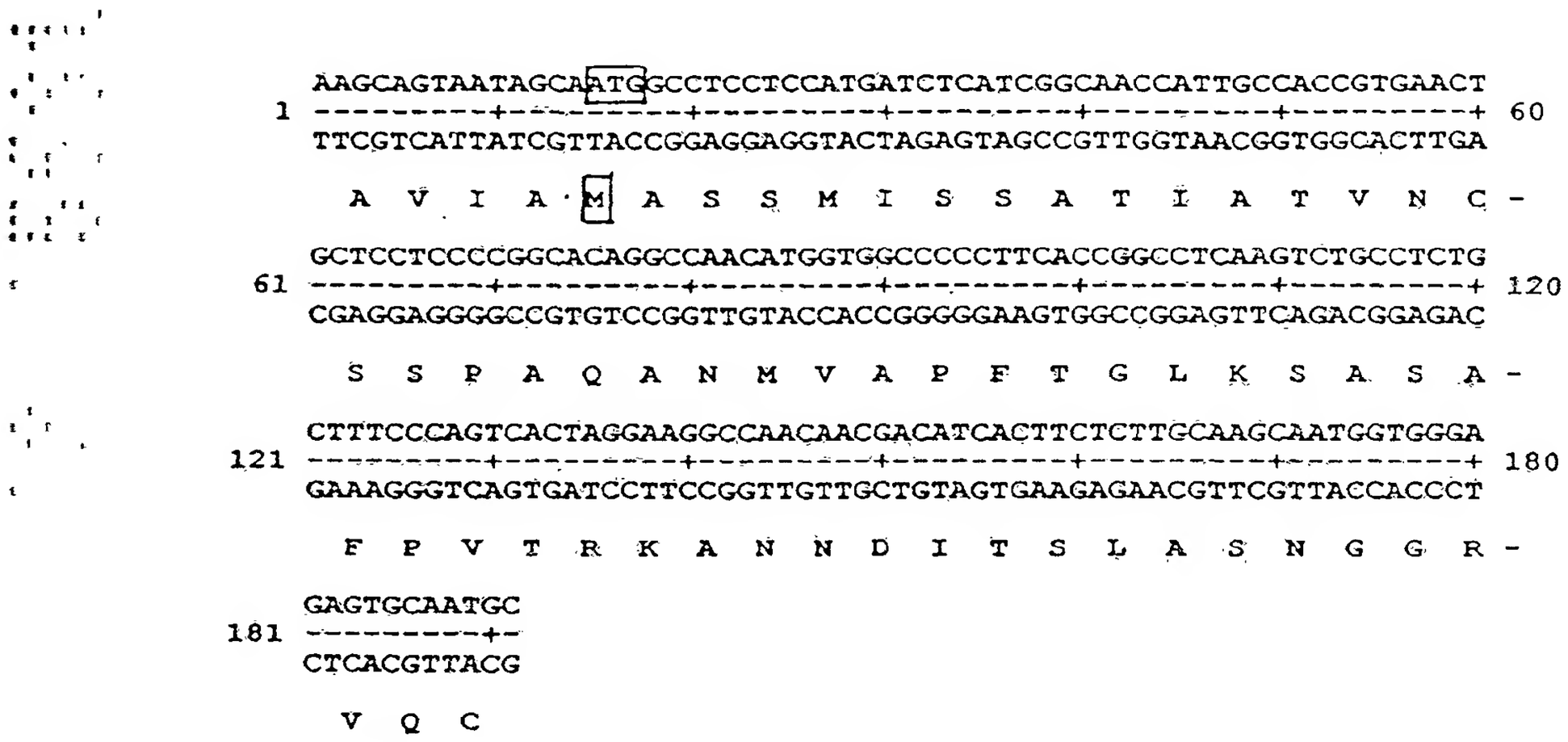

FIG.26